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Physiological Zoölogy

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Physiological Zoölogy

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THE INTRACELLULAR DIGESTION OF THYMUS NUCLEOPROTEIN IN TRICLAD FLATWORMS

(Two plates and one text figure)

EDWARD GILMAN KELLEY
University College, New York University

STUDIES of the digestive processes in the turbellarian flatworms have been carried on since the pioneer work by Metschnikoff (1866). Prominent among the names of the older workers are those of Metschnikoff (1866–1901), Arnold (1909), St. Hilaire (1910), and Lohner (1916). In more recent years, Westblad (1923) has made a comprehensive study of digestion and excretion in a number of turbellarian forms, using living specimens whenever possible. Willier, Hyman, and Rifenburgh (1925) have made a very complete and systematic study of intracellular digestion of liver in *Planaria dorotocephala*. They used the histological stains, iron haematoxylin and Mallory's triple stain for protein material, and osmic dichromate fixation and Sudan III for fat material. Various chemical reagents were also used.

In general, these workers have shown that digestion of food occurs in a definite sequence. This involves the formation of food vacuoles, in the distal ends of the phagocytic cells, containing fragmentary food particles which break up into fine granules and later condense to form homogeneous masses which disappear in the proximal end of the intestinal cells.

The present work is a study of the intracellular digestion of thymus nucleoprotein by means of histological stains and the

polarizing microscope. The histological investigation is similar to the work of Willier, Hyman, and Rifenburgh (1925) with iron haematoxylin, and in addition contains a study of nucleic acid digestion by means of the Feulgen "nucleal reaction." The investigation with the polarizing microscope is a study of crystal formation in the living animal, during the process of digestion.

The writer wishes to express his sincere thanks for the criticisms and helpful suggestions of Professor Daniel Ludwig.

METHODS

The study of the intracellular digestion of nucleoproteins was carried out on the turbellarian flatworm *Planaria dorotocephala*. A detailed description of the morphology of the worm was made by Hyman (1925). Willier, Hyman, and Rifenburgh (1925) state that the digestive processes in the worms used in their experiments required about 5 days. In order to assure almost complete absence of food material in the phagocytic cells of the worms used in these experiments, they were removed from the general culture dishes and starved for a period of from 10 to 11 days. Later evidence showed that this period was quite sufficient. Wulzen (1926) has studied nutrition of *Planaria maculata* by measuring the comparative growth rate with various foods. Both liver and thymus were shown to be excellent food, causing rapid growth and frequent reproduction by fission. The food material used in all experiments was the thymus gland of the calf, and was always obtained either freshly removed from the animal or in a thoroughly frozen condition. When cut in small pieces and placed in water, it presented an excellent feeding surface for the worms, one to which they could attach readily and tear off the cells with evident ease.

The method of feeding was much the same as that used by Willier, Hyman, and Rifenburgh (1925) in their experiments with liver. Pieces of thymus, about 1 inch square, were placed in a dish with about 50 starved worms, and the time of attachment of the worms was noted. Most of the worms attached almost at once, and the remainder within a few minutes. Two of the worms, whose exact time of attachment had been noted, were removed after 5 minutes and killed immediately. Likewise, 2 were removed at 30 minutes and

killed. The exact time of attachment of these 4 had to be noted because of the short feeding time allowed. With the remainder, the time of attachment was approximately correct within a few minutes. Worms were removed, as above, at the following intervals: 1, 3, 6, 18, 24, 30, 42, 50, and 65 hours, and 3, 4, 5, 6, 7, and 8 days after attachment to the food. The time required for the individual worms to complete feeding varied somewhat, the average being from 1 to 1½ hours. After 2 hours all the worms had left the food material, and it was removed from the dish. The water was changed at once and any remaining food was carefully washed out with fresh water. The dish was then put aside for removal of the worms as indicated above, the water being changed once each day until the completion of the experiment. The same procedure was carried out in all the feeding experiments. It gave a representative picture of the course of digestion.

Several methods of killing were tried: dropping the fixing fluid directly on the extended worm; dropping glacial acetic acid on the worm and then washing immediately; and dropping on it a 2 per cent solution of nitric acid and washing. The last method was the most satisfactory, since the worms were killed and remained fully extended. A worm was removed from the culture dish and placed in a moistened watch glass in front of a fairly strong beam of light, which caused it to move in a straight line away from the light. When fully extended, the 2 per cent HNO_3 was dropped on it and then washed off almost at once with distilled water, immediately after which the worm was removed to the fixing fluid.

The fixing fluid used on all of the sections was a saturated solution of mercuric chloride made up in 0.6 per cent sodium chloride. The worms were transferred to vials directly after killing and washing. The time allowed for fixing was from 2 to 3 hours. The remainder of the process followed the regular histological technique. Ten μ longitudinal sections were cut in both dorsoventral and horizontal planes, the former being the most satisfactory in the majority of cases. At least six slides of each worm were made, each containing a considerable number of sections cut well enough into the digestive tract to give a clear picture of the phagocytic cells in all regions of the body.

In order to find out whether the digestion of the almost pure nucleoprotein food proceeded in the same manner as the digestion of the liver proteins, complete series of slides were stained in iron haematoxylin and Mallory's triple stain, the two stains most used by Willier, Hyman, and Rifenburgh (1925). In 1924, Feulgen and Rossenbeck proposed the use of the Schiff's aldehyde (fuchsin-sulphurous) reagent for the staining of cell nuclear material. They worked out a technique for the application of this reagent to sectioned material, and chemical investigation indicated that it was a specific stain for nucleic acid which has been mildly hydrolyzed. In order to investigate the course of the digestion of the nucleic acid of the nucleoprotein food, this "nucleal reaction" was used on a complete series of slides. A number of workers have described the technique to be followed, but the procedure given by Ludford (1927) seemed to be the most concise and clearly worked out. This technique was followed in detail in the staining of the slides, giving excellent results in all cases. The sections were first hardened overnight in 90 per cent alcohol and then run down into water. One complete series of slides was hydrolyzed in HCl at 60° C. The sections were then stained, washed, and mounted in balsam. A second complete series was carried through the same procedure, but without hydrolyzing in HCl at 60° C. These sections were transferred directly from water into the fuchsin-sulphurous reagent. This series served a double purpose: to act as a check on the normal hydrolyzed sections and to show whether any of the nucleic acid in the phagocytic cells was being hydrolyzed by the worm. One complete series of hydrolyzed, stained sections and one series of unhydrolyzed sections were counterstained with an aqueous solution of light green. The slides were dipped and rinsed immediately in distilled water. Excess water was blown off, and absolute alcohol was poured on. The slides were differentiated and mounted in balsam.

To obtain some insight into the formation and use of the purine and pyrimidine bases of nucleic acid, a second series of experiments was conducted, using the polarizing microscope on the living planarian, since these bases are crystalline compounds insoluble in water. A Bausch and Lomb polarizing microscope was used, with a blue-yellow filter disc for greater color variation and more detail.

The examination was carried out as follows: A medium-sized worm was placed on a cover slip with a small amount of water. When the worm had righted itself, which it always does immediately, the cover slip was placed on a slide. The elongation of the worm caused the attracting force of the cover slip with the small amount of water to exert sufficient pressure to flatten it, ventral side up, without causing undue distortion or death. It could then be examined under both the low- and high-power lenses of the polarizing microscope. Preliminary experiments showed that culture worms, which had been fed on beef liver, contained in their digestive tract a considerable number of tiny crystals, seen as yellow gold specks against a dark background. As these were difficult to observe, because of the strong refraction of the muscle fibers,[1] an attempt was made to find some reagent which would dissolve out the crystalline structures of the muscles without dissolving the crystals of the digestive tract. It was found that glacial acetic acid, diluted by water in the tissues and to some extent that present on the slide, completely removed the color from the muscle fibers at once, leaving the tissue intact and quite transparent. It was impossible to find a worm without crystals present, but after 30 days' starvation the number was reduced to a few crystals in some of the more anterior branches, so few that any increase could easily be detected.

A considerable number of *Planaria* were removed from the general

[1] Large numbers of longitudinal red stripes were seen throughout the body of the worm and numerous red cross stripes could also be noted, depending upon the state of contraction of the worm in any one place. The pharynx was brilliantly striped with both longitudinal and cross fibers which were constantly changing from red to green and green to red as it moved. In a dark-blue field (prisms completely crossed) these fibers were white against the dark background. The location of the stripes showed that they were the longitudinal and circular muscle fibers of the body and the muscles of the pharynx.

According to Schipiloff, Danielewsky, and O. Nasse (see Landois and Stirling, 1891, p. 575), the contractile anisotropous substance of striated muscles consists of myosin, which occurs in a crystalline condition. Myosin is a globulin, soluble in dilute acids and alkalies. Of the two proteins of muscle fibers—myogen and myosin—only myosin is said to occur in the muscles of invertebrates. Johnstone (1923) states that smooth muscles also possess the property of double refraction, though the particles responsible for it are not arranged in the form of alternating dark and light bands. This information would indicate that the muscles of *Planaria dorotocephala* are smooth muscles, since no striations could be seen even under an oil-immersion lens. The doubly refractive substance is probably myosin, especially since tests showed that it was readily soluble in dilute acids and alkalies.

cultures and starved for a period of 30 days. The worms were then fed as noted above, and, instead of killing, they were placed under a cover slip and examined with the polarizing microscope. (Pieces of thymus, crushed under a cover slip, were also examined.) After this examination, the water was drawn off and glacial acetic acid was drawn under the cover slip. The worm was killed instantly, and was perfectly flattened without distortion. The cover slip could then be raised, and a drop of acetic acid was placed directly on the worm to assure complete penetration. A second examination was then made. Again the cover slip was raised and the tissue was washed with distilled water for a few minutes. A drop of dilute HCl was added, and the cover slip was replaced and an examination was made at once. This same procedure was used on all of the worms throughout the series, except that various reagents were substituted in testing solubilities of the crystals. The reagents used were as follows: concentrated and dilute solutions of hydrochloric acid (HCl), ammonium hydroxide (NH_4OH), sodium hydroxide (NaOH); dilute nitric acid (HNO_3); and alcohol of various strengths. Only one reagent was used on each worm, with the exception of acetic acid, which was added first in many cases.

OBSERVATIONS

SECTIONS STAINED WITH IRON HAEMATOXYLIN

Plate I, Figure 1, shows a typical section of *Planaria*, taken before feeding and after a starvation period of 11 days. The small intestinal cells have a highly vacuolated appearance. Only occasional deeply stained masses can be seen arising from the muscular layer of the intestine, probably gland cells. Food material was noticeable in the lumen in sections taken 5 minutes after feeding and showed as late as 6 hours after feeding in some of the branches of the gut (Plate I, Fig. 2). The process of vacuole formation and the condition of the food material in the vascuoles are shown in Table I and Plate I, Figures 2–6.

Regarding color changes in the sections of various stages, little more can be said than to note the gradual change from a lighter blue, through a blue gray in the granular stages, to a deep blue in the homogeneous spherules. A comparison with the work of Willier,

Hyman, and Rifenburgh (1925) shows that the digestion of the nucleoprotein material corresponds almost exactly with the digestion of the liver particles as noted in their slides stained with iron haematoxylin or Mallory's triple stain.

SECTIONS STAINED WITH FEULGEN'S REAGENT

Hydrolyzed material.—To compare the digestion of the protein material, as noted above, with the nucleic acid as shown by the

TABLE I

SECTIONS STAINED WITH IRON HAEMATOXYLIN

Time Killed after Feeding Started	Type of Vacuole				
	Fragmentary	Granular	Transitional	Homogeneous	Stained by Iron Haematoxylin
5 min.	Beginning	None	None	None	Very few
30 min.	Very numerous	Coarse	None	None	Increasing
1 hr. 15 min.	Very numerous	Fine beginning	None	None	Increasing
3 hr.	Very numerous	Many	Beginning	None	Increasing
6 hr.	Decreasing	Very numerous	Many	Beginning	Increasing
10 hr.	Few	Very numerous	Many	Many	Increasing
18 hr.	None	Decreasing	Very numerous	Very numerous	Nearly all
24 hr.	None	Few	Very numerous	Very numerous	Nearly all
30 hr.	None	None	Few	Very numerous	Decreasing
65 hr.	None	None	Occasional	Many	Decreasing
72 hr.	None	None	None	Few	Decreasing
8 days	None	None	None	Occasional	Almost none

Feulgen reagent, an examination of the slides of this series was made with the foregoing points in mind. Table II and Plate II, Figures 7–10, show the condition of the food material in the vacuoles as stained by this reagent. Comparison of almost exactly the same places on 24-hour, haematoxylin and hydrolyzed-fuchsin stained sections, cut only a few 10-μ sections from one another (Plate I, Fig. 6, and Plate II, Fig. 10), showed a large number of small solid spherules in all sections of the phagocytic cells in the iron haematoxylin slides (with possibly a greater number toward the basal end), while

PLATE I

ABBREVIATIONS

b., blue-stained spherule
d.v., diffuse vacuole
f., food material
f.v., food vacuole, fragment stage
g.c., gland cell
g.s., granular spherule
h.s., homogeneous spherule
l., lumen
n., nuclei
p., phagocytic cell
t.s., transitional spherule

FIG. 1.—View of intestinal wall and gut lumen of *Planaria dorotocephala*, showing clear and somewhat collapsed appearance of phagocytic cells before feeding, after a starvation period of 11 days (series PH_2). Longitudinal section, sublimate fixation, stained with iron haematoxylin. ×440.

FIG. 2.—Appearance of food material in lumen of gut and phagocytic cells 30 minutes after attachment to food (series $2H_2H$). Longitudinal section, sublimate fixation, iron haematoxylin. ×440.

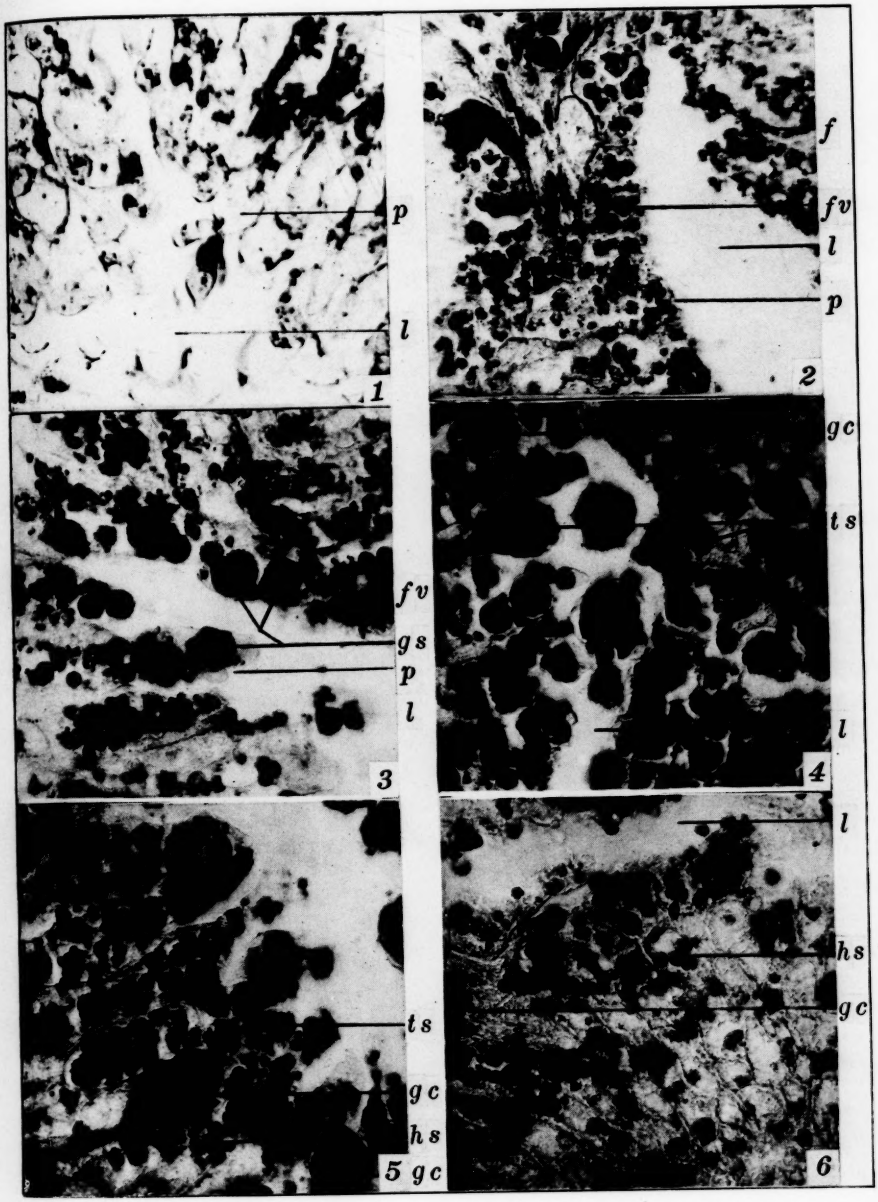
FIG. 3.—View of phagocytic cells, showing numerous large vacuoles filled with finely granulated food material (series $2H_4H$) 3 hours after attachment. Anterior end of longitudinal section. Sublimate fixation, stained with iron haematoxylin. ×440.

FIG. 4.—Phagocytic cells showing vacuoles containing numerous round deeply stained food particles, transitional spherules (series $2H_5H$) 6 hours after attachment to food. Longitudinal dorsoventral section (posterior end). Sublimate fixation, iron haematoxylin stain. ×440.

FIG. 5.—Early homogeneous spherule stage. Phagocytic cells contain many round deeply stained vacuoles with considerable size variation in different regions of cells. Transitional spherules still present and gland cells prominent (series $2H_5H$) 6 hours after feeding. Longitudinal dorsoventral section (anterior end). Sublimate fixation, stained with iron haematoxylin. ×440.

FIG. 6.—Appearance of phagocytic cells, in anterior section, 24 hours after feeding. Homogeneous spherules generally small and not as numerous. For comparison with fuchsin-stained section of same period (series $2H_7H$). Sublimate fixation, iron haematoxylin. ×440.

PLATE I



the fuchsin slides showed a greatly decreased number of vacuoles which stained red. The vacuoles which did stain appeared to be in the transitional or early homogeneous stages. Later slides showed this same disproportion of vacuoles in the two series; but the comparison became more difficult, since the number of homogeneous spherules decreased in the later sections of both series. These sections in which the majority of transitional spherules were found (18–30 hours) certainly indicated that there was much less staining of nucleic acid, as shown by the red color with the fuchsin reagent. This does not mean that there were fewer vacuoles, since many clear or granular vacuoles could be seen on the slides. It was the red staining which had decreased.

The question of a color change in the fuchsin-stained sections was rather carefully investigated to determine any change from a violet-red to a violet or blue color. The color of the vacuoles and food material of the early slides was a bright red-violet. In the later slides, the color may have been slightly more violet, but not sufficiently so to be anything other than a slight variation of the stain. The blue-violet and blue color, to be noted later under the section on counter-staining, was not present in this series of hydrolyzed slides strained with fuchsin alone. The nuclei of the body walls showed plainly as violet-red granular masses, and the color was constant throughout the series.

The appearance of the color in relation to the food particles was also carefully noted. In the early slides the color was definitely concentrated in the food particles and was not diffused throughout the vacuole. In the later slides there may have been some diffuse staining in the vacuoles, beginning with the 18- and 24-hour slides; but this was more or less confused by the appearance, at this stage, of the more evenly stained early homogeneous spherules. Comparison with both the iron haematoxylin slides and the unhydrolyzed fuchsin slides showed, however, that the staining was more diffuse in these slides than in the iron haematoxylin slides, but less diffuse than the staining of the vacuoles of the unhydrolyzed fuchsin sections.

Unhydrolyzed material.—Table II and Plate II, Figure 11, show the condition of sections stained in fuchsin reagent without previous

PLATE II

(For abbreviations, see explanation of Plate I)

FIG. 7.—Section of intestine showing food material in lumen and phagocytic cells as revealed by staining hydrolyzed sections with fuchsin-sulphurous reagent. Fragment stage (series $_2H_4F$). Posterior end of longitudinal section, 3 hours after attachment to food. Sublimate fixation. ×440.

FIG. 8.—Anterior end of same section (series $_2H_4F$) 3 hours after attachment to food, showing large grandular vacuoles in phagocytic cells. Sublimate fixation, hydrolyzed section, stained with fuchsin-sulphurous reagent. ×440.

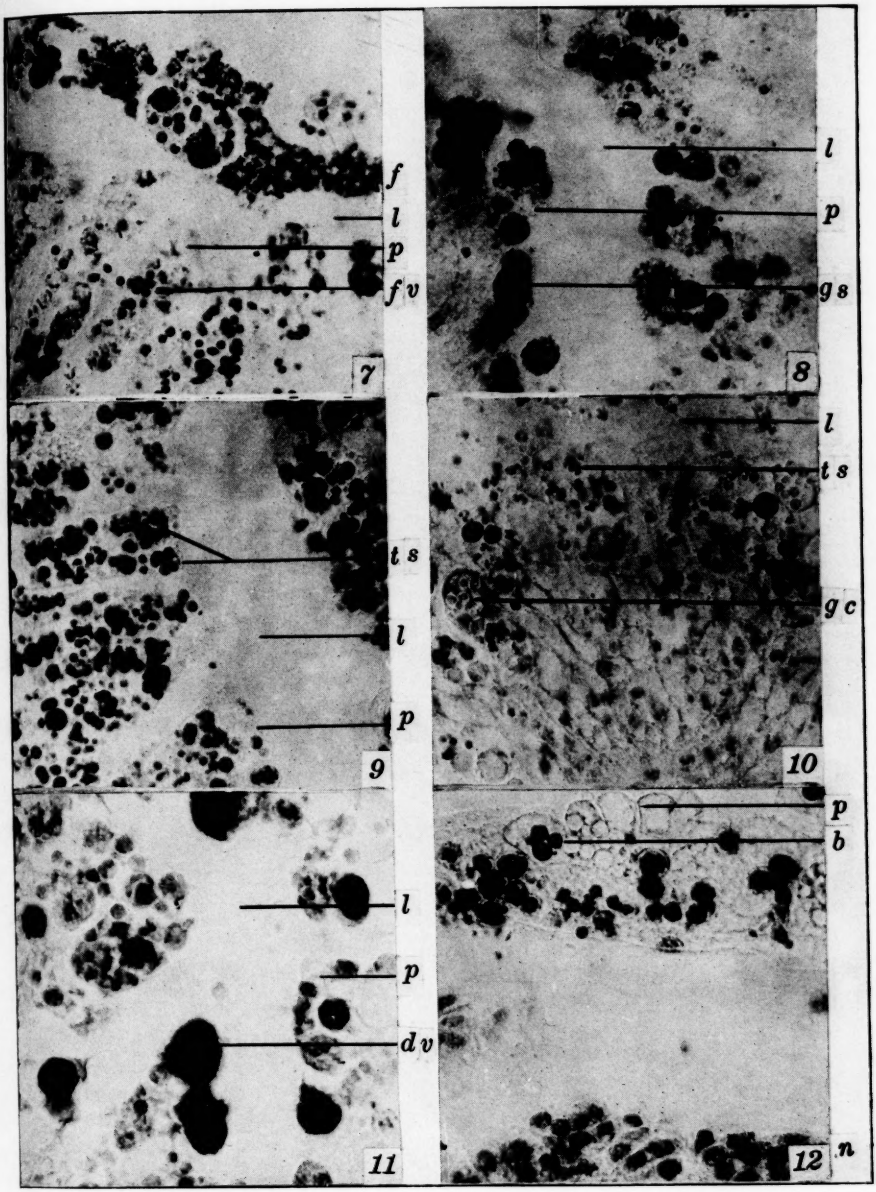
FIG. 9.—Fuchsin-stained section showing transitional vacuoles (series $_2H_6F$) 18 hours after feeding. Gland cells present but unstained. Sublimate fixation, hydrolyzed section, stained with fuchsin-sulphurous reagent ×440.

FIG. 10.—Appearance of phagocytic cells 24 hours after feeding, stained with fuchsin-sulphurous reagent (series $_2H_7F$). Exactly the same location as shown in Figure 6 (series $_2H_7H$) and separated from it by a few 10 μ sections. Stained material noticeably less in fuchsin-stained sections ×440.

FIG. 11.—Section of unhydrolyzed material, stained with fuchsin-sulphurous reagent. Shows phagocytic cells containing a few large diffusely stained vacuoles and some smaller less heavily stained ones (series $_2H_5Fu$). Longitudinal section, 6 hours after feeding. Sublimate fixation. ×440.

FIG. 12.—Posterior end of 65-hour section of hydrolyzed material, stained with fuchsin-sulphurous reagent, counterstained with light green. A few homogeneous spherules still present, the majority of which have a definite blue color. The nuclei, in the cell walls, have the characteristic red-violet color as seen in all sections of this series. ×440.

PLATE II



hydrolysis in HCl at 60° C. There was no staining of any material in the sections of the unfed worms. Although food material could be noted in the lumen of the gut, and even in the phagocytic cells of

TABLE II

SECTIONS STAINED WITH FUCHSIN-SULPHUROUS REAGENT

Time Killed after Feeding Started (See Table I)	Type of Vacuole				
	Fragmentary	Granular	Transitional	Homogeneous	Stained by Feulgen's Stain
	Sections Hydrolyzed in HCl at 60° C.				
5 min.	Beginning	None	None	None	Very few
30 min.	Numerous	Coarse	None	None	Increasing
1 hr. 15 min.	Very numerous	Fine beginning	None	None	Increasing
3 hr.	Very numerous	Many	Beginning	None	Increasing
6 hr.	Decreasing	Very numerous	Many	Beginning	Increasing
10 hr.	Few	Very numerous	Many	Occasional	Increasing
18 hr.	None	Few	Numerous	Occasional	Numerous
24 hr.	None	None	Occasional	Occasional	Rapidly decreasing
30 hr.	None	None	Few	Few	Almost none
65 hr.	None	None	None	Rarely	None
72 hr.	None	None	None	Rarely	None
	Sections Unhydrolyzed in HCl				
5 min.	None	None	None	None	None
30 min.	None	None	None	None	None
1 hr. 15 min.	Few	Few	None	None	Very few
3 hr.	Few	Many	None	None	Slowly increasing
6 hr.	Few	Numerous	Occasional	None	Numerous
10 hr.	None	Numerous	Occasional	Occasional	Numerous
18 hr.	None	Few	Occasional	Occasional	Rapidly decreasing
24 hr.	None	Very few	Few	Few	Rapidly decreasing
30 hr.	None	None	None	None	None
72 hr.	None	None	None	None	None

5-minute sections, there was again no evidence of staining in these sections. In 30-minute sections, there seemed to be the faintest tinge of a pink color in some of the food material in the lumen and in a few vacuoles of the phagocytic cells, but the color was so weak

and indefinite that no conclusion could be made regarding its presence. Three-hour sections, however, showed a definite staining reaction. In these sections there were numerous vacuoles, the great majority of which contained definitely red-stained granules. The granular vacuoles of this series appeared somewhat like the fragment granules noted in the hydrolyzed sections, but their color was a decidedly fainter red and they did not seem to have quite as definite outlines as the hydrolyzed material. Six-hour sections showed that a great many, but by no means all, of the vacuoles in each phagocytic cell were stained. It was unusual to find more than one or two in each cell which showed noticeable staining. The 6-hour vacuoles appeared to be mostly in the finely granular stage, and those vacuoles with color present showed a small number of granules with a fairly heavy red-orange color. The entire vacuole was stained, however, the appearance being that of a colored liquid inclosed within the vacuole wall. The depth and shade of color varied in the individual vacuoles. There were numerous large vacuoles stained a diffuse orange-red with a faint indication of a violet color. These vacuoles were almost entirely in the distal ends of the phagocytic cells. Other smaller vacuoles, both with and without red granules, had a much fainter red color, with a tendency toward a violet-red shade. These vacuoles were found both in the distal end and deeper down in the cell.

Eighteen-hour sections had much the same appearance as regards number of vacuoles stained and their color. There was no appearance of transitional spherules in these sections or in the 24-hour sections. The latter, however, showed a decided decrease in the number of stained vacuoles. Only an occasional large red vacuole could be seen, and the number of red-violet vacuoles was less, although many were still present. Thirty-hour sections showed only a few stained vacuoles, and in these the coloring was very faint. None of the later slides showed any definitely red-stained vacuoles, and the number of diffuse red-violet vacuoles was small. The nuclei of the body walls showed no staining reaction throughout the entire series of slides.

COUNTERSTAINING

Special mention must be made regarding the appearance of the fuchsin-stained sections counterstained with light green, because of

an unusual staining effect found in the vacuoles. All of the vacuoles in the sections up to 18 hours showed the characteristic red-violet color of the fuchsin reagent, with the usual green cytoplasmic stain of the light green. In 18-hour sections there was a faint tinge of a blue color in some of the more basal vacuoles, but almost all of the stained vacuoles were still a decided red color. In 24-hour sections, there was a noticeable number of vacuoles present which had a very definite blue color, many which had a blue-violet color, and a considerable number which were still a distinct red-violet. Thirty-hour sections showed a large number of blue vacuoles, with a number of the larger vacuoles still a definite red-violet color. From 30 hours on, the sections showed a majority of blue-stained vacuoles with red color still present in some of the larger ones. The blue color was located in the small homogeneous spherules or in the small spherules present in the transitional vacuoles (Plate II, Fig. 12). The depth of color varied, from a blue so faint that it could hardly be distinguished, to a decided deep sky blue. As previously noted, the fuchsin slides which were not counterstained with light green showed no blue color. Sections of unhydrolyzed material, stained with fuchsin and counterstained with light green, also showed no blue-colored vacuoles. Sections of various stages hydrolyzed in HCl at 60° C., stained with light green without previous staining in fuchsin, showed a considerable number of vacuoles in the later stages stained a much deeper green than the surrounding cytoplasm of the phagocytic cells. No green-stained vacuoles could be found in any of the slides stained with fuchsin and counterstained with light green. Sections stained in light green, without either previous hydrolysis or fuchsin staining, showed only the characteristic green cytoplasmic stain of the light green, with no deeper green vacuoles.

CRYSTAL FORMATION

Table III shows the course of the formation of crystalline material from the digestion of thymus nucleoprotein. The crystals appeared as very small glittering white specks. Although 1-hour specimens definitely showed crystals, it was not until 3 hours after feeding that the concentration had become sufficiently high to avoid confusion with those crystals which might have been present originally. The maximum concentration was reached at 30 hours. At this time the

TABLE III

FORMATION OF CRYSTALS

Time Killed after Feeding Started	Appearance of Intestine and Food Material	Appearance and Concentration of Crystals	Effect of Reagents on Crystals
5–10 min.	Highly distended; food mass dense, grayish; convolutions	Crystals, if present, obscured by food mass	
20–30 min.	More distended; food mass opaque; few convolutions	Few crystals visible; may have been those present originally	
1 hr.	Food mass less opaque	Definite increase	Insoluble in acetic acid. Soluble in concentrated and dilute HCl, NaOH and dilute HNO_3
3 hr.	Still highly distended; food mass much less opaque	Higher concentration	
6 hr.	Many transparent areas	Concentration increasing in all transparent areas	Slowly soluble in concentrated NH_4OH. One hour after this reagent had been added, crystals still present. X-shaped in contrast to specks shown before. (See text Figure A)
8 hr.	Convolutions; a great many transparent areas in anterior branches	High concentration in all transparent areas	
18 hr.	Convolutions in all regions; food mass gone	High concentration in all branches	
24 hr.	Decreasing in size	Very high concentration	
30 hr.	Further reduction; many more branches	Extremely high concentration	
48 hr.	Slow decrease in size	No decrease in concentration	
72 hr.	Slow decrease in size	No decrease in concentration	
96 hr.	Slow decrease in size	No decrease in concentration	
8 days		Number greatly decreased in most specimens; high concentration in many	A few crystals insoluble in HCl
18 days		Numerous crystals present throughout intestines of all worms	

crystals appear as a white gold mass dotted with black specks rather than a black mass dotted with crystals. No decrease in concentration could be noted as late as 96 hours after feeding. Very fresh thymus, when examined under the polarizing microscope, showed no crystals in the tissue of the gland, although the blood vessels did contain some doubly refractive substance. Pieces of thymus of various ages showed that considerable quantities of crystalline substances had developed in the tissues, quickly when the thymus was left at room temperature (within a day), but slowly even when the material was kept on ice.

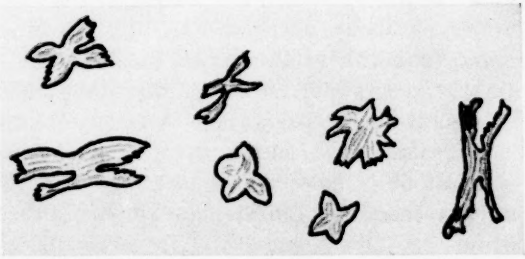


FIG. A.—Drawings of crystals which appear after the addition of concentrated ammonium hydroxide to a living worm which has fed on thymus. See Table III.

DISCUSSION

Nucleoproteins are composed of one or more protein groups combined with a phosphorous-containing substance, nucleic acid, by a relatively unstable linkage which may be compared to a salt linkage. The protein of thymus nucleoprotein, is considered almost pure histone, a strongly basic simple protein; and the nucleic acid is termed "animal nucleic acid," as distinguished from "plant nucleic acid." The structure of animal nucleic acid has been the subject of recent investigation by Levene and London (1929). These authors show that it is made up of four mononucleotides connected to one another through an oxygen bond between the carbohydrate groups and the phosphoric-acid molecules. Each mononucleotide of animal nucleic acid contains an ortho-phosphoric acid molecule and a carbohydrate group. Each of two mononucleotides contains a purine base, and each of the other two has a pyrimidine base. The purine bases of animal nucleic acid are guanine and adenine, and the pyrimidine bases are thymine and cytosine.

The exact nature of the carbohydrate compound has been the object of much study (Kossel, 1893; Feulgen, 1922 and 1924). Levene and Mori (1929) have compared the properties of the carbohydrate, which they term "thyminose," with those of ribodesose and xylodesose. In a later paper, Levene, Mikeska, and Mori (1930) definitely arrived at the conclusion that the carbohydrate of thymonucleic acid is d-2 ribodesose.

The action of iron haematoxylin on the nucleoproteins is apparently not definitely known. In a recent paper, Robertson (1929), referring to an 1898 paper by Mathews, makes the statement: "The constituents of the nucleus which are stained by hematoxylin are, as A. P. Mathews has shown, nucleic acids." Mathews (1926) states: "It is of interest to recall that all so-called nuclear stains of a basic nature, with the exception of the mordanted stains, such as iron hematoxylin, combine with nucleic acid." A variety of materials are stained by iron haematoxylin, such as cytoplasmic inclusions (yolk granules; fat granules; basophile, acidophile, and neutrophile granules; and mitochondria), centrosomes, chromatophores of protozoan cells, and the myelin sheaths of the brain. As previously noted, the protein of thymus nucleoprotein is histone, a strongly basic protein. Since the reaction of the mordant, iron alum, is acid in solution, it seems probable that if a chemical reaction were to occur, it would be between the mordant and the protein rather than between the mordant and the strongly acid nucleic acid. The view generally taken now is that the action of the mordanted stains, especially iron haematoxylin, is a physical adsorption phenomenon, and this view would more easily account for the variety of substances stained. If the staining of the nucleoproteins by iron haematoxylin is an adsorption phenomenon, it is possible that the nucleic acid might also be concerned; but it seems more probable that it is largely the protein which is stained. Further evidence to support this might be seen in the work of Willier, Hyman, and Rifenburgh (1925) on the nature of the solid spherules in the later stages of digestion of the protein food material. These solid spherules stained strongly with iron haematoxylin, and when tested with Millon's reagent and the xanthoproteic reaction, gave strong protein tests. Nucleic acid does not give positive tests for proteins with these reagents.

If the iron haematoxylin stain is an indication of proteins, it appears that the protein of the nucleoprotein passes through a definite sequence of digestion from fragmentary food particles, through granular and transitional stages, to form solid spherules, which decrease in size and disappear in the more basal ends of the phagocytic cells.

The chemistry of the Feulgen "nucleal reaction" is quite definitely established. This reaction is based on Schiff's aldehyde test, and is given by any aldehyde group. Basic fuchsin, a bright red-violet dye, is decolorized by the addition of sulphurous acid. On the addition of an aldehyde group, a colored fuchsin compound is formed. Wieland and Scheuing (1921) have worked out this reaction in great detail, and they show that the fuchsin-sulphurous reagent is N-sulphinic acid of para-fuchsin-leuco sulphonic acid. The reaction with aldehyde produces an ester which at once rearranges to give an imide derivative.

In excess of sulphurous acid this compound forms again the leucosulphonic acid, which is now active again to form a new N-sulphinic group and react with a second aldehyde group. The authors state that the dialdehyde compound is violet, whereas the monoaldehyde compound is a more red color. The dialdehyde compound is incapable of further reaction, and accordingly cannot become decolorized. Feulgen and Rossenbeck (1924) applied this test to the staining of nucleoproteins. Ludford (1927) states that, as a microchemical test, the "nucleal reaction" depends upon the presence of an aldehyde group among the products of a partial hydrolysis of thymus nucleic acid. In this partial hydrolysis, the purine bases—adenine and guanine—are separated off, leaving thymic acid, a more resistant and ill-defined substance. He states, "According to Feulgen, it is the thymic acid which contains the aldehyde group and gives the Schiffs reactions." An intense purple or violet coloration is the result. It seems quite evident, in view of the work of Levene, Mikeska, and Mori (1930), that the aldehyde group responsible for the color reaction with the fuchsin-sulphurous reagent is that present in the desoxy sugar, d-2 ribodesose. This sugar shows the typical aldehyde grouping on the 1-2 carbon atoms. Feulgen has pointed out that the fuchsin test is absolutely specific

for partly hydrolyzed animal nucleic acid. In the opinion of the author, this statement needs to be more definitely explained. It is certain that the test serves to distinguish animal nucleic acid from plant nucleic acid, since the plant nucleic acid produces no red color when treated in the manner prescribed by Feulgen. Nevertheless, the fuchsin-sulphurous reagent is acted upon by any aldehyde compound existing as such, or produced by the hydrolysis with HCl. Since Feulgen's statement about specificity seems to be well founded, it appears that the hydrolysis with HCl is either too mild to break down any of the tissue compounds other than animal nucleoprotein to an aldehyde group or is so drastic that it breaks down these compounds beyond the aldehyde stage. The strongest point in favor of this idea is the fact that only the cell nuclei are stained and the cytoplasm which might contain potential aldehyde groups is unstained.[1]

In view of Feulgen's work, it is safe to assume that the fuchsin-sulphurous stain gives an idea of the course of the nucleic acid digestion. Observation of the material previously hydrolyzed in HCl showed that the nucleic acid followed the same digestive changes as the protein, through the fragmentary and granular stages, but seemed to differ at the time the transitional spherules were forming, becoming noticeably different when these transitional spherules were changing to the solid spherule stage. This hydrolyzed material would not show when any actual digestion of the nucleic acid occurred, since the process of hydrolysis with HCl would cause the formation of the aldehyde group whether it existed in the nucleic acid combined with the protein, in the free nucleic acid, in a mononucleatide, or even in the thymic acid or free carbohydrate group. However, the absence of a staining effect would indicate that the thymic acid, or free carbohydrate group, had been broken down to such an extent that it no longer possessed the aldehyde group necessary in the staining reaction. Apparently this breakdown of nucleic acid did occur in the phagocytic cells of *Planaria*, and at an earlier stage than the breakdown of the protein material. There is a possi-

[1] A new characteristic color reaction of thymus nucleic acid has recently been described by Dische (1930). This is the color reaction with diphenylamine, and will be used in later experiments of the type given in this paper.

bility that the proteins themselves might be breaking down into peptid and polypeptid stages, or even into amino acids, and that these compounds might be giving the staining effect with iron haematoxylin. This latter possibility seems likely in view of the evidence of the early breakdown of the nucleic acid, although the two processes would not necessarily be the same. A breakdown of protein might even account for the greater intensity of the staining reaction with the iron haematoxylin.

If Feulgen's statement regarding the staining of only hydrolyzed nucleic acid is true, the formation of a red color in the vacuoles of the phagocytic cells of the unhydrolyzed material (unhydrolyzed in HCl at 60° C.) would indicate that hydrolysis of the nucleic acid was occurring in the vacuoles. The absence of a staining reaction in the cell nucleus proved that unhydrolyzed nucleoprotein material was not staining. Evidently, *Planaria dorotocephala* possesses enzymes capable of hydrolyzing nucleic acid into its constituents. The lack of a heavy staining reaction in these sections might indicate that hydrolysis is not very great, or that the compound is soluble and diffuses out, or that it is further broken down into a non-aldehyde compound so that there is no very great concentration of aldehyde present at any one time. The fact that the HCl-hydrolyzed sections showed a considerable amount of nucleic acid still present up to the transitional and solid spherule stages would indicate that hydrolysis is not rapid until these stages. The very diffuse nature of the stained material in the vacuoles would show that the aldehyde compound was soluble in the vacuole fluid. On the other hand, the absence of any staining reaction in the cytoplasm just outside the vacuoles would favor the idea that complete hydrolysis to a non-aldehyde compound was occurring in the vacuoles, rather than a diffusion of material out of the vacuole. It does not seem likely that there would be an immediate change from an aldehyde compound to a non-aldehyde compound simply by the passage out from the vacuole into the cytoplasm.

The greater intensity of stain in the large vacuoles of the unhydrolyzed sections, with a decreasing intensity in the smaller and more basal granules, would seem to show that hydrolysis, of the small amount of material that is being hydrolyzed by the vacuole

fluid, proceeds to the aldehyde stage in the large finely granular vacuoles, while the change of the vacuoles to a smaller transitional and solid stage brings about a more active hydrolysis to a non-aldehyde compound. It seems strange that no diffuse staining could be seen in the large finely granular vacuoles of the hydrolyzed sections. There is the possibility here that the HCl (at 60° C.) was sufficiently strong to complete the hydrolysis of the already partially hydrolyzed material to a non-aldehyde stage, while not sufficient to completely change the undigested nucleic acid during the short time allowed for hydrolysis.

The various shades of color shown in the stained vacuoles form an interesting part of this study of nucleoprotein digestion, but their explanation must necessarily be somewhat incomplete until the new study of chromolysis and the chemistry of the dyes is more thoroughly worked out. The decidedly more red color of the unhydrolyzed (in HCl) vacuoles, in the distal ends of the phagocytic cells, is interesting in the light of Wermel's statements on the more red color of the monoaldehyde fuchsin compound as compared to the violet-red color of the dialdehyde compound. This fact would support the idea already proposed, that the concentration of the aldehyde compound in these vacuoles was not very great compared to that produced by the hydrolysis of the nucleic acid by HCl. This study of monoaldehyde and dialdehyde compounds might also explain why some of the smaller vacuoles in the unhydrolyzed sections showed a violet color, by assuming a more rapid formation of aldehyde at any one time, as the vacuoles decreased in size. It is difficult to correlate this violet color, if due to an increase of aldehyde, with the fact that the less intensity of the staining indicates a decrease; but it is hoped that later work may clear up this point. The fact that the hydrolyzed series of slides did not show a great variation in shades of color might be expected, since the reagent was controlled as carefully as possible for each section, and any hydrolysis of unchanged nucleic acid would give much the same color in the later sections as it would in the earlier ones.

The most unusual color effect occurred when light green was used as a counterstain on the hydrolyzed fuchsin-stained sections. The blue color found in the later sections was so very definitely blue that

it could not be confused with any red-violet or violet color of the fuchsin stain itself; and, as mentioned in the observations, plain fuchsin slides without counterstaining did not show this blue color. On the other hand, sections, hydrolyzed with HCl and stained only with light green, did not show a blue color, although the vacuoles in the later slides did appear to have a greater affinity for the light-green stain. There are, then, two possible explanations for the formation of the blue color: that it was due to a mechanical color combination of the fuchsin and the light green, or that it was due to some chemical change in the vacuole contents which effected a change in the two dyes to produce a blue-colored dye. As the earlier counterstained sections did not show a blue color, the idea of a purely mechanical combination is not likely. A color combination of the fuchsin with the more intense light green found in the vacuoles of the later sections is possible, but this more intense light green indicates some change of the vacuole contents in relation to the stain. Numerous phagocytic cells contained both blue- and red-stained vacuoles, and this would again indicate a lack of a purely mechanical combination but would not rule out the combination with the more intense light green. The possibility of a chemical change in the dyes, when in combination with the vacuole contents, does not seem too remote when the structures of the dyes are taken into consideration. Both dyes are triphenyl-methane derivatives with numerous possibilities for additions and changes in structure. For instance, the entrance of phenyl groups into the rosanilin molecule produces the dyes anilin blue, and in another combination, spirit blue. The structure of methyl blue is similar to that of light green and offers a possibility of a change. Methyl and ethyl green, which are dyes capable of staining the vacuole material, are also triphenyl-methane derivatives; and the staining of the vacuoles by light green might be possible through their formation.

Although this discussion presents many unsolved problems, it seems as if the process of intracellular digestion of various types of food material might provide a further method for the study of the chemistry of staining. Later work will be undertaken on this line of investigation.

A complete interpretation of the observations on crystal forma-

tion cannot be made at this time, since these experiments were conducted with the view of noting if crystal formation did occur, when it occurred to the greatest extent, and when a decrease in the number of crystals took place. The preliminary solubility tests were made to try to obtain some insight into the nature of the crystals.

It can definitely be concluded that the digestion of nucleoprotein food material gives rise to large numbers of crystals in the phagocytic cells of the planarian intestine.

A time comparison of the formation of crystals with the formation of the different types of vacuoles is difficult, since the formation of the crystals was a gradually increasing process with no apparent stages. The 3- to 6-hour stained sections showed a majority of large finely granular vacuoles. Crystals, formed from the nucleoprotein, were present at this time and increased in number through the transitional and early solid spherule stages. It can also be recalled that the decrease in staining reaction of the hydrolyzed nucleic acid with basic fuchsin occurred at the period from 24 to 30 hours, the time when the greatest concentration of crystals was present in the phagocytic cells. The decrease in the amount of nucleic acid evidently corresponds with the increase in the number of crystals. It is also apparent that the decrease in the crystal content of the cells bears no relation to the decrease in the stained material, since almost all of the stained material was gone by 96 hours, and at this stage there was still the same high concentration of crystals found in the 30-hour worms.

The possibility of the crystals arising from histone (the protein of the thymus) seems unlikely. In the first place, the protein itself is soluble in water while the crystals were not—or at least not in the vacuole fluid. In the second place, the only crystalline amino acid found in histone which is insoluble in water is tyrosine, and it is readily soluble in ammonium hydroxide while the crystals were not. The intermediate products of protein hydrolysis, such as the peptones and proteoses, are so variable in character that little definite can be said about them. However, it seems likely that these products would have disappeared before the decrease in crystals occurred, since little staining of protein material could be found in the later stages.

Many of the products of nucleic acid hydrolysis are crystalline substances, with solubilities much like those found in these preliminary tests. Nucleic acid is a white powder, insoluble in alcohol and ether but soluble in alkalies and ammonia; it is insoluble in water, forming a slimy mass. This would seem to rule out nucleic acid itself as a source of the crystals in the phagocytic cells. The products of hydrolysis of nucleic acid are mononucleotides, nucleosides, carbohydrate phosphate complexes, carbohydrates, phosphoric acid, and the purine and pyrimidine bases. One or more of these products must be responsible for the appearance of crystals. Both of the purine bases—adenine and guanine—are crystalline compounds, insoluble in water. Adenine is soluble in acetic acid; and since the crystals were not soluble in acetic acid, this would indicate that the majority of the observed crystals were not adenine. Guanine, on the other hand, is not listed as soluble in acetic acid but is readily soluble in dilute mineral acids and bases, and is very slowly soluble in ammonium hydroxide. These were the same solubilities as were noted for the crystals. The two pyrimidines—cytosine and thymine —are also crystalline compounds insoluble in water, but the preliminary tests as made above were not sufficient to distinguish these compounds with any degree of certainty. The same is true of the carbohydrate compound, although it seems likely that this compound is soluble in water. The pure nucleoside of guanine with the carbohydrate molecule can be crystallized, as reported by Levene and Mori (1929), but whether this occurs during hydrolysis in the intestine is still to be determined.

The formation of the X-shaped crystals, when ammonium hydroxide was added to the specimens, is not of very great significance as yet. The only crystalline structures, found in texts on biochemistry, which correspond at all with the X-shaped crystals observed in this work, were the triple phosphate crystals, as shown by Hawk and Bergeim (1926). The formation of ammonium-magnesium phosphate seems possible since the nucleic acid contains considerable phosphoric acid, and the tissue of many invertebrates is said to be rich in magnesium compounds, especially when the culture medium is spring water. The formation of this compound might explain the greater quantity of these crystals in the early stages by assuming

that the phosphoric acid was still largely combined, so that its concentration when released was sufficient to give a reaction with ammonium hydroxide.

The nature of the enzymes which caused the formation of the crystals is questionable. As stated, fresh thymus contained no crystals; but thymus which had been allowed to age somewhat showed many crystals when examined with polarized light. Evidently, the thymus contained enzymes capable of forming crystalline compounds from nucleic acid. These enzymes may have been the cause of the hydrolysis of nucleic acid in the planarian intestine; but they alone could not have been responsible, since the hydrolysis in the worm was much more rapid than that in thymus left at room temperature. Also, the concentration of crystals in the phagocytic cells was much greater than that found at any time in the tissue of the thymus.

One other point must be mentioned regarding the nature of the crystals formed by the hydrolysis of the nucleoprotein. No evidence could be found that any of the oxidized compounds of the purine bases were formed in the phagocytic cells. Xanthine and hypoxanthine are quite soluble in ammonium hydroxide, whereas all of the crystals of the intestinal cells were very slowly soluble in this reagent, both in the early and late specimens. Uric acid, the other oxidized purine compound, is not soluble in hydrochloric acid; and, with the exception of a very few crystals in the later stages, all of the crystals were readily soluble in this reagent.

SUMMARY

A study of the digestion of thymus nucleoprotein in the intestine of the turbellarian flatworm *Planaria dorotocephala* has been made by means of histological strains and the polarizing microscope.

A series of sections of worms, fixed at various periods after feeding, was stained with iron haematoxylin, and the course of digestion was noted. Stages were found involving the formation of food vacuoles containing fragments of food material, the breaking-down of these fragments to form finely granular vacuoles, and the condensing of the fine granules to give, first, transitional spherules, and later, homogeneous spherules.

A second series of sections from the same worms was hydrolyzed in hydrocholoric acid at 60° C. and stained with fuchsin-sulphurous reagent, according to Feulgen's method. These sections showed the presence of nucleic acid in the vacuoles at the various stages. The course of digestion, as shown by this stain, appeared to be the same as that shown by the iron haematoxylin up to the transitional and early homogeneous stages; but at this time the amount of material stained by the fuchsin reagent became greatly reduced as compared with the amount stained by iron haematoxylin. It is concluded that the aldehyde compound of the nucleic acid, responsible for the reaction with the fuchsin sulphurous reagent, disappeared at an earlier stage than the material stained by iron haematoxylin, which is assumed to be protein material.

A third series of sections was stained with fuchsin-sulphurous reagent without previous hydrolysis in HCl, in order to show the presence of an aldehyde product of nucleic acid, hydrolyzed by the vacuole fluid itself. Very definite staining was found, but in a smaller number of vacuoles. The large finely granular vacuoles gave the most intense staining reaction, but even in these the color was much less intense than that of the hydrolyzed sections. Later sections showed even less color. This would indicate that hydrolysis of nucleic acid occurred in the early stages and that the aldehyde compound formed by this hydrolysis either was further acted upon to form a non-aldehyde compound or diffused out of the vacuole.

Other series of both hydrolyzed and unhydrolyzed sections were stained with fuchsin-sulphurous reagent and counterstained with light green. Hydrolyzed sections, beginning at about 24–30 hours, showed numerous blue-stained vacuoles. This blue color must have been due to a chemical change in the relation of the two dyes and the vacuole contents, since it was not shown by plain fuchsin-stained sections, or by plain light-green-stained sections, or by unhydrolyzed stained and counterstained sections.

The study of crystal formation, with the polarizing microscope, in relation to the digestion of thymus nucleoprotein, showed that gradually increasing numbers of crystals were formed in the phagocytic cells of the intestine from the beginning of digestion to about 30 hours after attachment, when a high maximum concentration was

reached. This high concentration persisted long after the disappearance of stained material, as shown by sections of the same number of hours, and was not noticeably decreased until 8 days after feeding. Preliminary solubility tests indicated that the crystals were the unoxidized purine or pyrimidine bases.

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STUDIES ON THE SEXUAL CYCLE IN BIRDS. V. EFFECTS OF LIGHT OF DIFFERENT INTENSITIES UPON THE TESTIS ACTIVITY OF THE EUROPEAN STARLING (STURNUS VULGARIS)[1]

(Three plates)

THOMAS HUME BISSONNETTE
Trinity College, Hartford, Connecticut

BISSONNETTE (1930*a*, 1931) has shown that increasing or decreasing the length of daily periods of illumination, by the use of 60-watt bulbs after dark, caused progressive or regressive germ-cell changes, respectively, in the testes of these birds. The changes were conditioned by relative daily periods and not by absolute length of period. They duplicate those in nature, except in time of inception and rate of reaction of germ-cell elements. He used both gradual and sudden increases in extra light time, up to from 6 to $7\frac{1}{2}$ hours nightly, to produce progressive changes, and sudden decreases of either daylight or electric-light periods to produce regressive changes. He showed that the testes of birds kept in the same cage together, undergoing the same light treatment for about a month, behave differently if their previous light history has been different. They become active if previous light periods have been shorter, and regress toward the quiescent state if their previous light periods have been longer than those in force at the time of common treatment. All his tests were made with light of the same approximate intensity from 60-watt electric-light bulbs.

Experiments have been carried out to test the effects of lights of different intensities upon the germ-cell activity of males of this same species, and their results are reported in this paper. In general, they show that lights from bulbs varying in strength from 10 to 60 watts are capable of inducing progressive testis changes when used, as were those in previous experiments, as added light given after sundown. However, the effects produced are not of the same magnitude, and

[1] Aided by a grant from the Committee on Research in Problems of Sex of the National Research Council.

the rate of acceleration of germ-cell activity is not the same, with different intensities of added light. The rate of increase in activity varies with the strength of bulb or light intensity from 10-watt to 25-watt bulbs and possibly to 40 watts, but above that for 50- and 60-watt bulbs there was no constant appreciable difference, unless perhaps the 50-watt bulbs were slightly more effective than the 60-watt ones. Tests were made of the effects of 60-watt bulbs both with and without forced muscular exercise. As before, the effect of added exercise is to delay the effect of light or cause testes to remain smaller than under conditions otherwise the same. Juvenile birds (those of the previous summer's broods) do not have as great increases in testis size as do mature birds under the same light treatments. The total testis size produced in the middle of winter by artificial light stimulation to activity is at least as great as normal, and, in one or two cases, apparently even greater.

MATERIAL AND METHODS

The birds used in these experiments were European starlings (*Sturnus vulgaris*) caught in the same place as those used in previous experiments and studies (Bissonnette, 1930*a*, 1931). The manner of catching them was the same except that a wooden box with a wire screen front and sliding door was used in place of a canvas bag to hold the birds as caught. Both males and females were mixed indiscriminately in all cages. There was no preliminary period in captivity before beginning experiments, to permit the birds to become accustomed to captivity, as before.

Eight cages were used in the same room, as before described. All cages, except that with the motor-driven belts and sweeps, had 12 birds, and this "work" cage had 11 birds only. So the experiments began with 95 birds, indiscriminately mixed as to sexes, in 8 cages.

Some refinements in the methods of caring for the birds were made possible by the grant from the Committee on Research in Problems of Sex of the National Research Council and are worth mentioning. The food consisted, as before, of scraps from the kitchen of the College Commons, ground up in a large, motor-driven meat-chopper, and mixed with corn meal and bran. Small boxes containing sand were placed in the cages and flat pans with water for baths.

Drinking fountains of the types used for baby chicks, consisting of a fruit jar inverted over a star-shaped open pan, kept the drinking water relatively clean. Baths could be taken without loss of drinking water. The food trays of the sort previously described were supported at or above the levels of the roosts, instead of being placed on the floors of the cages as was previously done. This lessened the pollution of food by droppings from roosting birds.

As in previous experiments, light after sundown was directed into each cage, except the control cage and those with the 60-watt bulbs, from a bulb of the desired strength held in an improvised reflector made by removing about one-third of the side of a tomato can and perforating the end to admit the socket for the bulb. These reflectors were fastened to the wire mesh at one side of the cages just above the level of the roosts at a distance of about 9 inches from the middle of the roost.

All cages bore as nearly the same relation as possible to daylight entering the basement room by three large windows (Bissonnette, 1931). Cages were not completely curtained off while the added light was being given; so a dim diffuse light spread over the room. Apparently this did not affect the controls appreciably, as is shown by comparison of inside and outside controls at the end of the experiment.

One cage of birds was used as control, and it was so curtained that no direct light from any of the bulbs reached it. The other cages had 10-, 15-, 25-, 40-, 50-watt bulbs respectively, and two other cages received light from a single 60-watt bulb hung between them in such a way as to give the same intensity of light into each. One of these cages was equipped to cause its birds to take forced exercise because of belts and motor-driven sweeps described in previous papers (Bissonnette, 1930*a*, 1931). The types of cross-pieces used as sweeps in this cage were changed so that each sweep consisted of two wooden strips crossing each other at right angles and fastened at their ends to the belts at such an angle as to prevent or minimize "creep" of one belt past the other on the pulleys. This prevented stopping of the belts by the strips becoming caught. An electric time switch was used to turn on and off all the lights and the motor at exactly the desired times. This eliminated the timing variations

characteristic of the previous experiments when this was controlled by the night watchman at his convenience. The relative intensities of the lights in the various cages may be seen from the following table of data obtained from the Hartford Electric Light Company.

60 watts = 678 lumens	25 watts = 240 lumens
50 watts = 525 lumens	15 watts = 131 lumens
40 watts = 408 lumens	10 watts = 71 lumens

A uniform point source of 1-candle intensity emits 4π lumens, or 1 lumen $= \frac{1}{4\pi}$-candle intensities.

On November 9, 1929, lights and motor were run for 30 minutes after dark; November 19, 40 minutes; November 11, 1 hour; November 12, 1 hour 20 minutes; November 13, 1 hour 40 minutes; November 14, 2 hours; November 15, 2 hours 20 minutes; November 16, 2 hours 45 minutes; November 17, 3 hours; and from that date rising 15 minutes per day to 6 hours nightly. Thereafter it was kept constant till January 27, when the experiment terminated. In the meantime daylight time per day decreased from November 9 to December 21 normally for this latitude, and then slowly increased to the end of the experiment (Bissonnette, 1931).

Food trays, water vessels, and sand boxes were removed from both the 60-watt cages from 4:30 P.M. until 7:00 A.M. nightly, but not from the other cages. Except for this, all birds had food before them during daylight in excess of their needs. The room was kept cool by having the windows raised a little from the bottom all the time.

There were no deaths of any birds from any cause during the whole time of experiments from November 9 to January 27. Birds were killed and gonads examined and fixed on December 13, 23, and January 27, and a few birds were carried over into the experiments with colored light which followed with a new set of birds. Fixation was with Bouin's fluid with formic acid in place of acetic. Testes were cut from paraffin 7.5 μ thick, stained with iron haematoxylin and mounted in balsam. Study was made by direct observation of sections with the microscope and from comparison of photomicrographs of comparable regions of all testes made to the same magnifi-

cation of 335 diameters and $3\frac{1}{4}\times4\frac{1}{4}$ inches in size. This gives accurate comparisons of tubule size, relative number of germ-cell elements, interstitial cells, and pigment masses.

TABLE I

GONADS OF BIRDS KILLED DECEMBER 13, 1929; IN EXPERIMENTAL CAGES FOR 34 DAYS

Light Strength	Age	Gonad Size (Very Approximate) in Mm.	Gonad Color	Remarks on Plumage, Etc.
Control	M.*	R—2.4×1.6 L—3.0×1.5	Dark green	Plumage and bill mature autumn condition
	Y.*	R— as above L— as above	Dark green	Plumage and bill juvenile
10 watts	M.	R—2.8×2.6 L—3.4×2.4	Green	Plumage and bill mature autumn condition
	M.	R—3.8×2.8 L—4.3×2.7	Green	As above
15 watts	M.	R—4.1×3.6 L—5.0×3.4	Green	As above
	2 Y.	R—3.9×3.7 L—4.5×3.4	Green	Bill and plumage juvenile
25 watts	M.	R—6.8×6.0 L—9.2×5.9	Light green	Bill and plumage mature autumn condition
	Y.	R—5.0×3.7 L—5.4×3.8	Light green	Bill and plumage juvenile
	Y.	R—5.6×4.9 L—6.8×4.7	Light green	As above
40 watts	M.	R—7.0×5.3 L—7.9×4.8	Gray	Bill and plumage mature autumn condition
	2 Y.	R—4.8×4.4 L—6.5×3.8	Light green	Bill and plumage juvenile
50 watts	M.	R—7.0×5.8 L—9.0×5.7	Gray	Bill and plumage mature autumn condition
	Y.	R—not found L—7.4×4.5	Gray	Bill and plumage juvenile
60 watts without work	M.	R—6.2×5.5 L—8.2×5.1	Gray	Bill and plumage mature autumn condition
	Y.	R—as above L—as above	Gray	Bill and plumage juvenile
60 watts plus work	M.	R—7.3×5.0 L—8.5×5.2	Gray	Bill and plumage mature autumn condition
	2 Y.	R—5.2×5.4 L—6.3×3.8	Green	Bill and plumage juvenile
Totals	9 M. 11 Y.			

*M. = Mature individual: Y. = Juvenile individual.

Table I gives data regarding birds killed December 13, 1929, including wattage of lights used, sex of birds, whether mature or juvenile, gonad size, gonad color, and number of days in cage. Measures of testis size for right and left testes were from free-hand drawings made at killing, and are therefore only roughly approximate.

TABLE II

GONADS OF BIRDS KILLED DECEMBER 23, 1929; IN EXPERIMENTAL CAGES FOR 44 DAYS

Light Strength	Age	Gonad Size, Measured in Mm.	Color	Remarks on Bill and Plumage
Control	M.	R—2.5×1.7 L—3.2×1.6	Dark green	Bill and plumage mature autumn
	Y.	R—as above L—as above	Dark green	Bill and plumage juvenile
10 watts	3 Y.	R—6.0×4.8 L—7.9×4.6	Light green	Bill and plumage juvenile
15 watts	Y.	R—6.7×6.0 L—9.7×5.6	Light green	As above
	Y.	R—6.7×5.5 L—7.6×5.4	Light green	As above
25 watts	M.	R—6.7×5.4 L—8.5×5.3	Light green	Bill and plumage mature autumn
	Y.	R—7.6×7.3 L—10.4×7.0	Light green	Bill and plumage juvenile
	Y.	R—6.3×4.5 L—7.1×4.0	Light green	As above
40 watts	M.	R—9.6×7.7 L—11.3×7.4	Gray	Bill and plumage mature autumn
	Y.	R—7.0×5.9 L—7.5×4.5	Green	Bill and plumage juvenile
50 watts	M.	R—8.5×7.7 L—11.4×8.1	Gray	Bill and plumage mature autumn
	Y.	R—9.8×8.7 L—11.5×8.7	Gray	Bill and plumage juvenile
60 watts alone	M.	R—8.4×6.4 L—9.5×6.0	Gray	Bill and plumage mature autumn
	2 Y.	R—6.0×5.0 L—7.0×4.4	Green	Bill and plumage juvenile
60 watts plus work	2 M.	R—7.2×6.1 L—9.1×6.1	Green	Bill and plumage mature autumn
	2 Y.	R—5.3×4.7 L—6.9×4.2	Green	Bill and plumage juvenile
Totals	7 M. 14 Y.			

TABLE III

GONADS OF BIRDS KILLED JANUARY 27, 1930; IN EXPERIMENTAL CAGES FOR 79 DAYS

Light Strength	Age	Gonad Size in Mm.	Gonad Color	Remarks on Plumage, Etc.
Inside control	M.	R—3.0×2.3 L—3.8×2.1	Green	Bill and plumage autumn mature
	Y.	R—2.6×1.8 L—2.7×1.7	Green	Bill and plumage juvenile
Outside control	M.	R—2.6×1.9 L—3.2×1.8	Green	Plumage mature; bill yellow
	M.	R—3.1×2.6 L—3.9×2.1	Green	As above
	M.	R—3.3×2.0 L—3.4×1.8	Green	As above
	M.	R—2.8×1.9 L—3.4×1.7	Green	As above
	M.	R—3.2×2.4 L—3.6×2.5	Green	As above
	M.	R—2.4×1.7 L—2.8×1.5	Green	As above
	Y.	R—1.8×1.8 L—2.2×1.5	Green	Plumage juvenile; bill yellowing
10 watts	M.	R—9.0×6.6 L—9.8×6.1	Gray	Plumage and bill mature
	Y.	R—3.5×2.4 L—4.3×2.3	Green	Plumage and bill juvenile
15 watts	M.	R—15.4×11.8 L—16.2×9.8	Gray	Bill and plumage in breeding stage
	Y.	R—9.2×6.4 L—10.4×5.9	Gray	Bill and plumage juvenile
25 watts	M.	R—10.6×7.0 L—10.9×6.6	Gray	Bill and plumage in breeding stage
	M.	R—12.5×9.2 L—13.8×7.7	Gray	As above
40 watts	M.	R—14.0×10.9 L—16.8×8.8	Gray	As above
	Y.	R—3.6×2.2 L—4.2×1.9	Green	Bill and plumage juvenile
	Y.	R—2.8×2.2 L—4.0×1.7	Green	As above
50 watts	M.	R—17.2×12.7 L—16.6×10.5	White	Breeding bill and plumage; right gonad largest both ways
	Y.	R—17.6×11.3 L—18.7×9.9	White	Breeding plumage; bill dark
60 watts no work	M.	R—14.2×10.8 L—14.9×9.8	White	Bill and plumage breeding condition
	M.	R—14.6×10.6 L—15.4×9.0	White	As above
Totals	15 M. 7 Y.			

Table II gives like data for birds killed December 23, 1929; and Table III, for birds killed January 27, 1930, including outside controls, except that in this table the dimensions of all testes were measured with calipers to tenths of a millimeter. Bill color and plumage condition are included in remarks. Sexes can be distinguished, on inspection, by the color of the base of the mandible when it cannot otherwise even be guessed. In the male, the expanded portion of this region is pale blue, while in females it is yellow or pink. In juvenile birds it is more difficult to discriminate because the whole bill is covered by a dark layer which somewhat obscures this difference.

RESULTS AS SHOWN BY MICROSCOPICAL EXAMINATION

1. *Birds Treated from November 9 to December 13, 1929—34 Days*

a) *Inside controls.*—Young inside controls have testes which have remained in the normal midwinter condition with the three types of nuclei within the tubules, described by Bissonnette (1930*a*, 1930*b*, 1931) and Bissonnette and Chapnick (1930). Interstitial cells are numerous in the spaces between the tubules, where pigment is present in scattered flakes. Both testes and tubules are very small in diameter (Plate I, Fig. 1).

Mature birds from the same cage (Plate I, Fig. 2) have larger testes and wider tubules, but the conditions within the tubules are very similar. Pigmentation is much more dense and forms two rows of spots between tubules. Interstitial cells are numerous.

b) *10-watt bulb.*—The testes of the two mature birds killed from this cage are noticeably larger than those of controls of comparable age (Table I). One (Plate I, Fig. 3) is but little more advanced in spermatogenesis than the mature control (Plate I, Fig. 2). There are more nuclei of the large round type, the tubules are larger in diameter, and a few synizesis stages of primary spermatocytes are found in the tubules. Interstitial cells are relatively less numerous.

The testis from the other mature bird, subjected to light from a 10-watt bulb for 34 days, has increased a little more in size. Its tubules are much larger (Plate I, Fig. 4), and in many of them synizesis stages are very numerous. In others they are not so

PLATE I

All figures are photomicrographs made at 335 diameters from testis cross-sections and reduced to ×223 approximately. Exact testis from which each figure is taken is in black-faced type.

FIG. 1.—Testis No. **1**. Young "inside control," treated from November 9 to December 13 (34 days).

FIG. 2.—Testis No. **2**. Mature "inside control," treated from November 9 to December 13 (34 days).

FIG. 3.—Testis No. **3**. Mature "10-watt" bird, treated from November 9 to December 13 (34 days).

FIG. 4.—Testis No. **4**. Mature "10-watt" bird, treated from November 9 to December 13 (34 days).

FIG. 5.—Testis No. **5**. Young "15-watt" bird, treated from November 9 to December 13 (34 days).

FIG. 6.—Testes Nos. 6 and **13**. Mature "15-watt" and "50-watt" birds, treated from November 9 to December 13 (34 days).

FIG. 7.—Testes Nos. **7** and 11. Young "25-watt" and "49-watt" birds, treated from November 9 to December 13 (34 days).

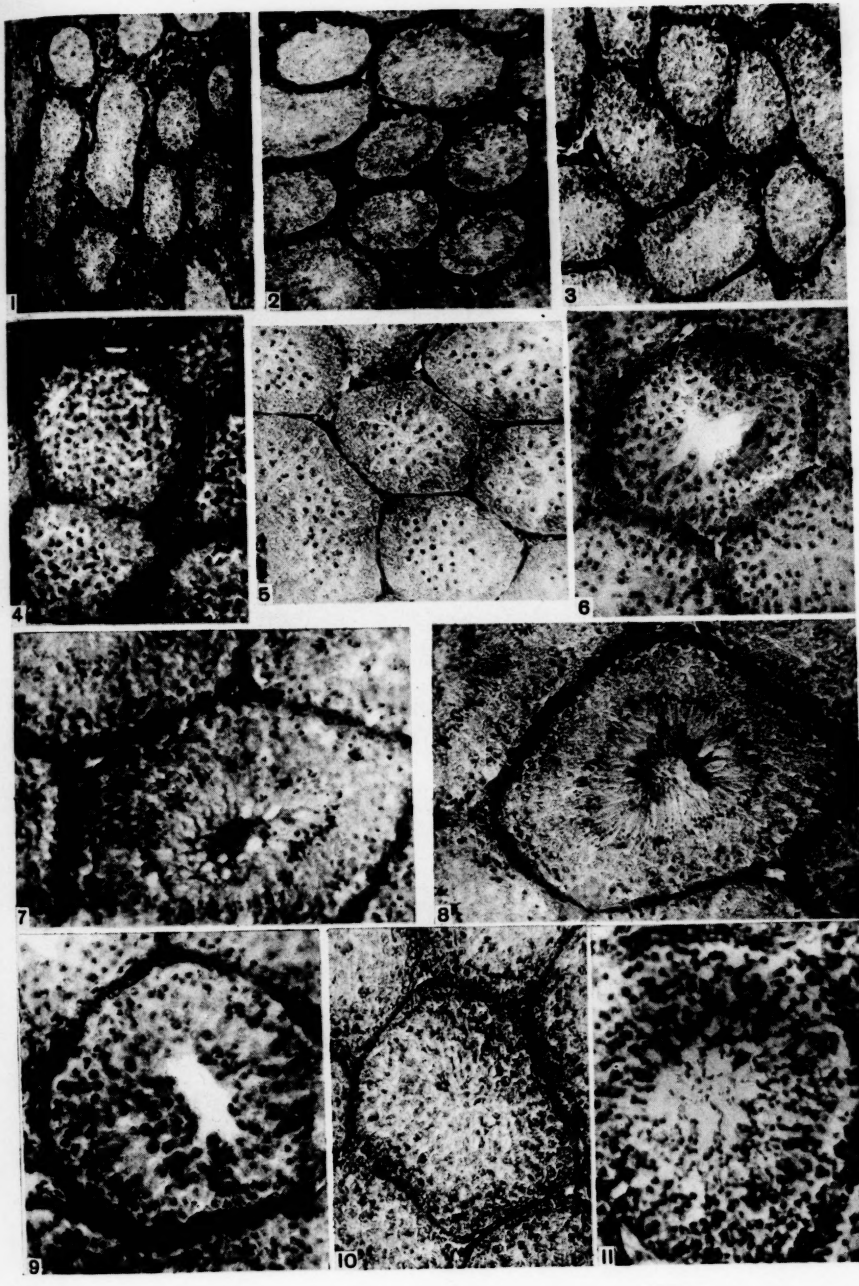
FIG. 8.—Testes Nos. 8 and **9**. Young and mature "25-watt" birds, treated from November 9 to December 13 (34 days).

FIG. 9.—Testes Nos. **10** and 15. Young "40-watt" and "60-watt without work" birds, treated from November 9 to December 13 (34 days).

FIG. 10.—Testes Nos. **12** and 14. Mature "40-watt" and young "50-watt" birds, treated from November 9 to December 13 (34 days).

FIG. 11.—Testis No. **16**. Mature "60-watt without work" bird, treated from November 9 to December 13 (34 days).

PLATE I



numerous. There is no definite localization of these different sorts of tubules, but both are scattered throughout the testis. Pigment is spread apart, and interstitial cells are difficult to distinguish, apparently being transformed into connective tissue cells under stress of tubule expansion as described by Bissonnette (1930*a*, 1930*b*) and Bissonnette and Chapnick (1930).

It is possible that the differences above described, between birds of comparable age and treatment, may be due to different points of habitual roosting in the cage, one nearer the light than the other. Though such constant differences of habit have not been noted during the experiments, while the birds have been under rather constant observation, the possibility must be noted as a possible cause of noted deviations.

c) *15-watt bulb.*—Testes of young birds (Plate I, Fig. 5) are much larger than those of even mature birds subjected to 10-watt bulbs. However, they show synizesis stages in fewer numbers than do those of the most advanced mature males subject to 10-watt bulbs. Pigment is more flattened out between tubules, and interstitial cells have almost all changed to connective-tissue-type cells. Many tubules show a lumen.

Mature birds with like treatment (Plate I, Fig. 6) have much larger testes, more scattered pigment, and no discernible interstitial cells. Some completed primary spermatocytes, as well as synizesis stages, are present. Most tubules have lumina. There is a more pronounced effect upon mature birds than on young ones of the previous summer's broods.

d) *25-watt bulb.*—Testes of young birds (Plate I, Figs. 7, 8), subjected to light from 25-watt bulb for the same period, have spermatids undergoing spermiogenesis, but no matured sperms as yet. Wavy tails on the sperms project into the lumina of the tubules. Pigment is much more scattered and tubules much larger than in any testes previously described in this paper. Testes are also larger than those subjected to less intense light. Interstitials are indistinguishable, and the cells of the tunicae propriae are flattened. All stages of spermatogenesis are shown.

Mature birds' testes (Plate I, Fig. 8) are larger than those of juveniles and closely approach maximum size. Their sperms are

more advanced in spermiogenesis. Otherwise they are not changed from conditions described for juvenile birds under like conditions. The cytoplasm of Sertoli cells inclosing clumps of sperms and spermatids is evident in many tubules.

e) *40-watt bulb.*—Testes of young birds vary from a condition (Plate I, Fig. 9) similar to that shown in Plate I, Figure 6, for mature birds subject to light from a 15-watt bulb, with primary spermatocytes and a few secondary spermatocytes but no spermatids nor sperms, to a condition (Plate I, Fig. 7) with immature sperms like that shown by some young birds subject to 25-watt bulbs (Plate I, Fig. 8). This may be due to variation in regular roosting place in the cage, as before suggested, or to individual idiosyncrasies of susceptibility. Sertoli cell cytoplasm incloses spermatids undergoing spermiogenesis (Plate I, Fig. 7).

Testes of mature birds (Plate I, Fig. 10) from the same cage are larger than those of young birds, but the tubules are not so far advanced in spermatogenesis as are those of one of the young ones. All stages up to spermatids are shown but no sperms except in a very few tubules. These sperms are in a very early stage of spermiogenesis. Interstitial cells and pigment are in the same condition as described for testes resulting from treatment with 25-watt bulbs.

f) *50-watt bulb.*—Testes of young birds subject to light from bulbs of this strength (Plate I, Fig. 10) are about two-thirds maximum size and show all stages of spermatogenesis up to and including very young sperms in which the tail filament is not yet well formed. Pigment is quite dense and tubules are not near maximum size.

Testes of mature birds from the same cage (Plate I, Fig. 6) are not so far advanced. They have no stages of spermatogenesis beyond primary spermatocytes. Tubules have lumina and pigment, and interstitial cells are in the condition described for mature males subject to 15-watt bulbs (Plate I, Fig. 6).

g) *60-watt bulb without forced exercise.*—Testes of young birds (Plate I, Fig. 10) show all stages up to secondary spermatocytes and are a little farther advanced than mature birds subject to 50-watt bulbs, but not so far as some young birds subject to the same treatment (50-watt bulbs). Testes are about two-thirds maximum size,

and tubules are larger than any in birds subject to 50-watt bulbs. Pigmentation and interstitial cells are unchanged from their condition under 25-, 40-, and 50-watt bulbs.

Testes of mature birds (Plate I, Fig. 11) are larger than those of young birds subject to the same treatment in this cage. Tubules are much larger, and a few very immature sperm heads are present. Except for the much larger tubules and more scattered pigment, these testes resemble very closely those of the young bird (Plate I Fig. 10) subject to a 50-watt bulb. Sertoli cell cytoplasm incloses spermatid nuclei and the very minute sperm heads.

h) 60-watt bulb with forced exercise.—Testes of young birds (Plate II, Figs. 12, 13) are smaller than those of birds subject to 60-watt bulbs without forced exercise, and some have their tubules less far advanced and lacking stages beyond primary spermatocytes (Plate II, Fig. 13). Others (Plate II, Fig. 12) show primary and secondary spermatocytes but neither spermatids nor sperms. Pigmentation and interstitial cells are unchanged from the conditions just described for 50- and 60-watt-bulb treatments.

There is an apparent lag in onset of spermatogenesis similar to that described by Bissonnette (1931) in birds subject to normal daylight with added periods of forced exercise but without added periods of artificial illumination. In the cases described by him, the initial lag was followed by more rapid changes induced by the added treatments till "worked" birds overtook and surpassed "unworked" birds at about 2 months.

Testes of mature birds subjected to "work" as well as 60-watt-bulb treatment (Plate II, Fig. 14) are about two-thirds maximum size and show all stages of spermatogenesis up to and including fairly mature sperms. Tubules are more advanced than any of those previously examined in this study. Spermatogenic activity, which possibly lagged at onset, has already surpassed that of birds with the same light treatment but without added "work."

Aside from this last described bird, those subject to light from the 25-watt bulb are more advanced in spermatogenesis than any others.

PLATE II

FIG. 12.—Testis No. **17.** Young "60-watt plus work" bird, treated from November 9 to December 13 (34 days).

FIG. 13.—Testis No. **18.** Young "60-watt plus work" bird, treated from November 9 to December 13 (34 days).

FIG. 14.—Testis No. 19. Mature "60-watt plus work" bird, treated from November 9 to December 13 (34 days).

Testes Nos. 23 and 24. Young "10-watt" birds, treated from November 9 to December 23 (44 days).

Testes Nos. 25 and 26. Young "15-watt" birds, treated from November 9 to December 23 (44 days).

Testes Nos. 28 and **29.** Young "25-watt" birds, treated from November 9 to December 23 (44 days).

Testes Nos. 30 and 31. Young and mature "40-watt" birds, treated from November 9 to December 23 (44 days).

Testes Nos. 56 and 57. Mature "40-watt" and "50-watt" birds, treated from November 9 to January 27 (79 days).

FIG. 15.—Testis No. **20.** Mature "inside control" bird, treated from November 9 to December 23 (44 days).

FIG. 16.—Testis No. **21.** Young "inside control" bird, treated from November 9 to December 23 (44 days).

FIG. 17.—Testis No. **22.** Young "10-watt" bird, treated from November 9 to December 23 (44 days).

FIG. 18.—Testis No. **27.** Mature "25-watt" bird, treated from November 9 to December 23 (44 days).

FIG. 19.—Testes Nos. 32 and **34.** Young "50-watt" and mature "60-watt without work" birds, treated from November 9 to December 23 (44 days).

Testes Nos. 33 and 35. Mature "50-watt" and young "60-watt without work" birds, treated from November 9 to December 23 (44 days).

FIG. 20.—Testis No. **38.** Young "60-watt plus work" bird, treated from November 9 to December 23 (44 days).

FIG. 21.—Testes Nos. 35 and 39. Young "60-watt without work" and mature "60-watt plus work" birds, treated from November 9 to December 23 (44 days).

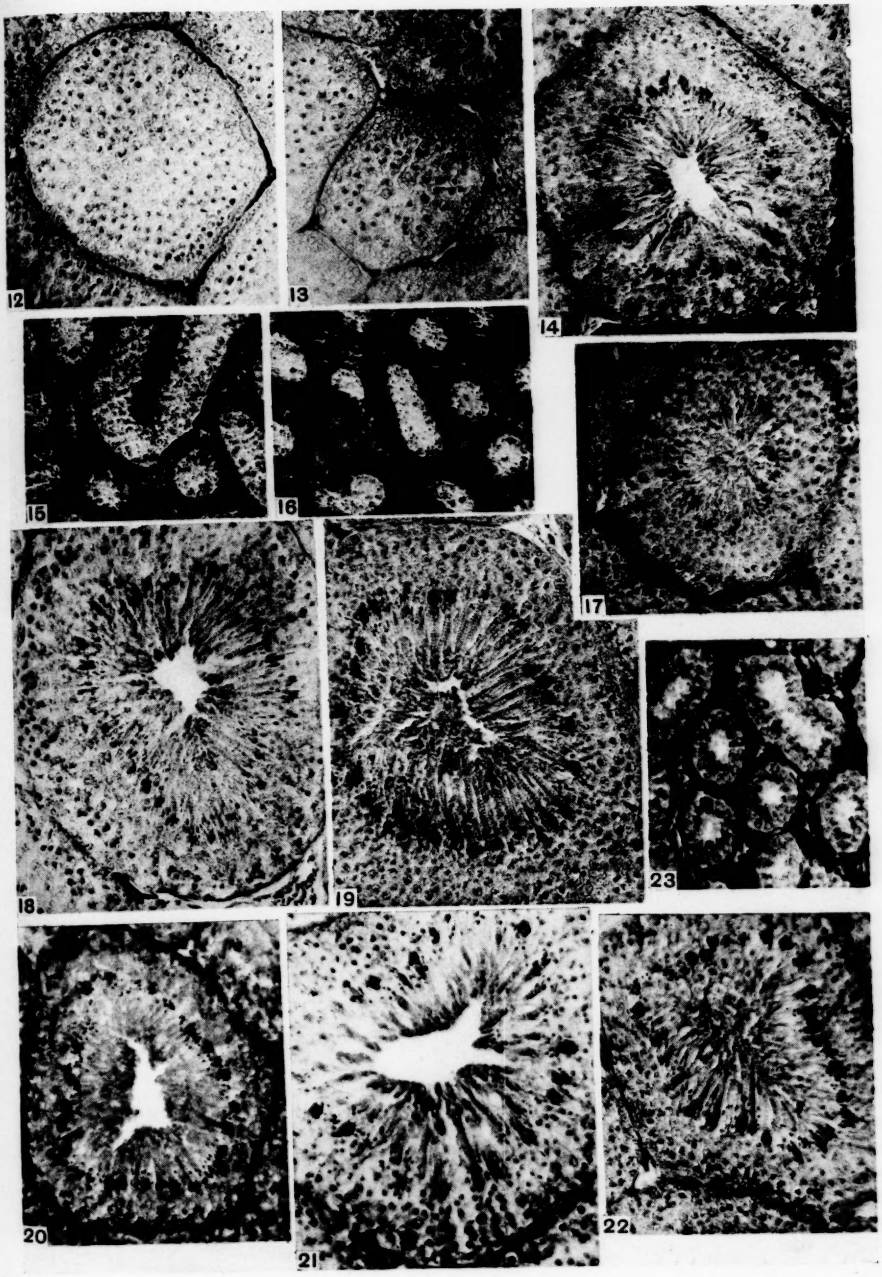
Testes Nos. 50 and 53. Mature "15- and 25-watt" birds, treated from November 9 to January 27 (79 days).

Testes Nos. 58, **59,** and 60. Young "50-watt" and 2 mature "60-watt without work" birds, treated from November 9 to January 27 (79 days).

FIG. 22.—Testis No. **37.** Young "60-watt without work" bird, treated from November 9 to December 23 (44 days).

FIG. 23.—Testes Nos. **40** and 43. "Outside control" birds, killed January 28.

PLATE II



2. *Birds Treated from November 9 to December 23, 1929—44 Days*

a) *Inside controls.*—Testes of both young (Plate II, Fig. 16) and mature birds (Plate II, Fig. 15) are very minute, those of mature birds slightly larger than those of young ones. In both, pigmentation is very dense and tubules very small indeed—those of young birds the smaller. Three types of nuclei are present in the tubules of both, and the larger round nuclei are relatively few in number. Tubules are smaller than those of the inside controls killed December 13, and pigmentation much denser. Testes of mature and of juvenile birds also differ much less than previously.

b) *10-watt bulb.*—Testes of young birds (Plate II, Figs. 14, 17) are at about two-thirds maximum size and show complete spermatogenesis, including more or less mature sperms. Pigment is scattered, and interstitial cells converted into connective tissue. These testes are as far advanced as those of mature birds subject to 25-watt-bulb or to 60-watt-bulb treatment were on December 13. This indicates that response is slower than that of birds subjected to stronger light; but testes of birds subjected to added light from 10-watt bulbs reach complete spermatogenesis in about 7 weeks, even in midwinter. While the rate of progressive change varies somewhat with the strength of light, it reaches the same state of complete spermatogenesis, finally, at the same time that controls are still undergoing regression.

c) *15-watt bulb.*—Testes of young birds (Plate II, Fig. 14) are between two-thirds and three-quarters maximum size. So far as can be determined, spermatogenesis is complete. Tubules are larger than in birds just described, subjected to light from a 10-watt bulb for the same time. Sperms appear more mature. These testes are still slightly in advance of those subjected to 10-watt bulb, and much farther advanced than birds from the same cage 10 days previously.

d) *25-watt bulb.*—Testes of a mature bird (Plate II, Fig. 18) are at about three-quarters maximum size, and show complete spermatogenesis, scattered pigment, and no discernible interstitial cells. Testes from young birds (Plate II, Fig. 14) have reached about two-thirds maximum size with tubules of somewhat smaller diameter;

but spermatogenesis is also complete. They, also, have advanced very much in the last 10 days.

e) *40-watt bulb.*—Testes of young birds are about three-quarters maximum size, with complete spermatogenesis (Plate II, Fig. 14) as described above. Mature birds' testes (Plate II, Fig. 14) are about maximum size, with complete spermatogenesis. Later stages of spermatogenesis are more numerous than in young birds from the same cage. Tubules are more distended. All testes on this date are much farther advanced than 10 days previously.

f) *50-watt bulb.*—Testes of both mature (Plate II, Fig. 19) and young birds (Plate II, Fig. 20) are about maximum size. Those of the young bird are larger than those of mature birds, and larger even than any ever seen in nature by the writer. All show complete spermatogenesis with abundant sperms, scattered pigment, and interstitials all transformed into connective tissue cells. While the testes of this young bird are the largest from starlings ever examined by the writer, their tubules are not so large in diameter as those of some other birds described. These testes also have advanced beyond those from the same cage on December 13, in both size and spermatogenic activity.

g) *60-watt bulb without "work."*—Testes of mature birds (Plate II, Fig. 19) and of young birds (Plate II, Figs. 10, 21) are in complete spermatogenesis. Those of mature birds are about maximum size, and those of the young birds at about two-thirds maximum. Tubules in mature birds are also larger than in young ones, and contain relatively more cells in the later stages of spermatogenesis. Otherwise the testes are alike. They have advanced in the last ten days.

h) *60-watt bulb with added "work."*—Testes of mature birds (Plate II, Fig. 21), at maximum size, and of young birds (Plate II, Figs. 20, 22), at about two-thirds maximum; all show complete spermatogenesis. Young birds are much farther advanced than those from the same cage 10 days previously. Mature birds show but little change in the same time, except in size of tubule and increase in numbers of sperms and spermatids. Pigmentation and interstitial cells are as before described for testes at complete activity.

3. *Birds Treated from November 9 to January 27, 28, 1930—79 days*

a) *Outside controls* (*Figs. 23, 24, 25*).—Testes of birds from outside in nature on these two days are small, those of young birds smaller than those of mature ones (Table III). All show some signs of increasing activity and some have mitotic figures (Plate III, Fig. 25). Tubules have enlarged somewhat over the midwinter minimum condition as described by Bissonnette and Chapnick (1930). Interstitial cells are still distinguishable and pigmentation is moderately dense in all cases. Three types of nuclei, described by Bissonnette and Chapnick (1930), are present; and the large round ones are relatively more numerous than in the midwinter testis condition, at which time the small round and oval ones predominate. No synizesis stages are present, and spermatogonial divisions are the only ones found.

b) *Inside controls, January 27, 1930.*—Testes of mature birds (Plate III, Fig. 27) have tubules enlarging with enlargement of testes (Table III). Mitotic figures and more numerous large round nuclei (spermatogonia) are present. Pigmentation is less dense, and pigment is stretched in strands round the tubules. Interstitial cells are still distinguishable in "nests" in the angles between tubules. Some are flattening out into connective tissue type nuclei. These testes show the effects of normally lengthening day as described by Bissonnette and Chapnick (1930).

Testes of young birds (Plate III, Fig. 28) also show activity within the tubules, which have increased in size since December 23, five weeks earlier. Pigmentation is more dense than in the mature birds' testes of this date, and the whole testes are smaller.

c) *Inside control bird* (*Plate III, Fig. 26*) *killed January 18, 1930.*—Testes are very minute, like those of inside controls on December 23, but with slightly larger tubules and more numerous nuclei within them. Pigmentation is very heavy, and interstitial cells are numerous. (This bird was killed because he showed signs of lessened activity or illness.)

d) *10-watt bulb.*—Testes of young bird (Plate III, Fig. 29) show signs of regression, with necrotic nuclei in the lumina of tubules, as well as mitotic figures among the spermatogonial layers. Pigmen-

PLATE III

FIG. 24.—Testes Nos. 41 and 42. "Outside control" birds, killed January 28.

FIG. 25.—Testis No. 44. "Outside control" bird, killed January 28.

FIG. 26.—Testis No. 45. Mature "inside control" bird, treated from November 9 to January 18 (70 days).

FIG. 27.—Testis No. 46. Mature "inside control" bird, treated from November 9 to January 27 (79 days).

FIG. 28.—Testis No. 47. Young "inside control" bird, treated from November 9 to January 27 (79 days).

FIG. 29.—Testis No. 48. Young "10-watt" bird, treated from November 9 to January 27 (79 days).

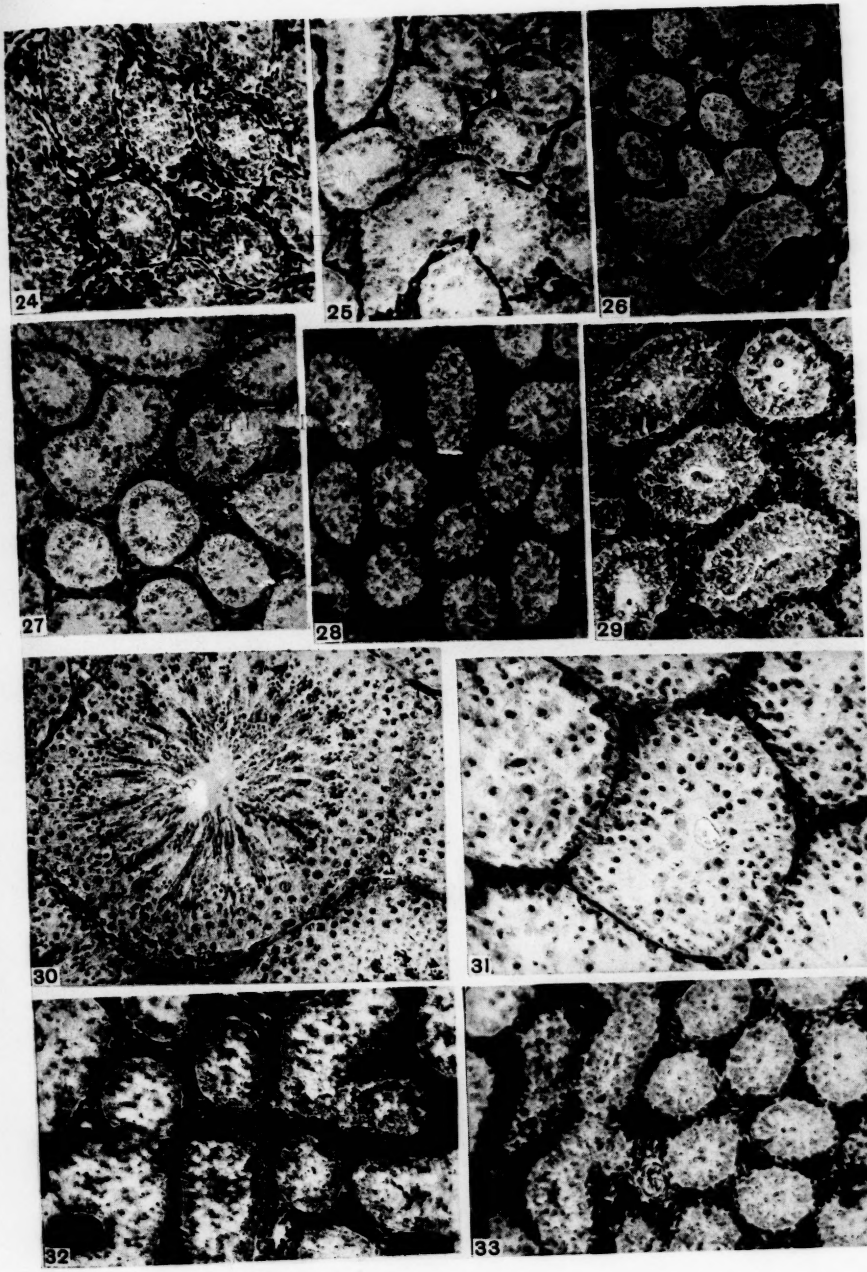
FIG. 30.—Testes Nos. 49 and 51. Mature "10-watt" and young "15-watt" birds, treated from November 9 to January 27 (79 days).

FIG. 31.—Testis No. 52. Young "25-watt" bird, treated from November 9 to January 27 (79 days).

FIG. 32.—Testis No. 54. Young "40-watt" bird, treated from November 9 to January 27 (79 days).

FIG. 33.—Testis No. 55. Young "40-watt" bird, treated from November 9 to January 27 (79 days).

PLATE III



tation is very dense, and interstitial cells are distinguishable. Cytoplasm in the tubules is vacuolated. Both testes and tubules are smaller than on December 23, when complete spermatogenesis was found in the tubules. This regression may be due to change of roosting place in the cage to a place receiving less light intensity, or to an individual idiosyncrasy of reaction. Day is lengthening for these birds.

Testes of mature birds (Plate III, Fig. 30) show complete spermatogenesis, large tubules, mature sperms (some of which are free in the tubules), scattered pigment, and interstitial cells indistinguishable from connective tissue. No signs of regression appear.

e) 15-watt bulb.—Testes of mature birds (Plate II, Fig. 21) are at about maximum size (Table III). Spermatogenesis is complete, with no signs of regression from the condition found on December 23, 1929. Testes of young birds (Plate III, Fig. 30) also show complete spermatogenesis in somewhat smaller testes. No regression or change from December 23 is evident.

f) 25-watt bulb.—Testes of young birds (Plate III, Fig. 31) show marked regression from their condition on December 23, 1929. Vacuolation of cytoplasm within the tubules and loss of all stages of germ-cells beyond synizesis have occurred. Tubules and testes are reduced in diameter. Pigment has aggregated into clumps in the angles between tubules, and some few interstitial cells have rounded up and are distinguishable from the connective tissue between the tubules. Sertoli cell cytoplasm is very plainly shown in sections. It surrounds the nuclei of germ-cells in various stages of spermatogenesis. Mitotic figures are present in spermatogonial and primary spermatocyte layers.

On December 23, just 34 days previously, testes of young birds from this cage were in complete spermatogenesis. This regression has occurred during a period in which daily artificial light periods were constant and daily daylight periods were slowly lengthening normally for this period.

Testes of mature birds (Plate II, Fig. 21) have shrunken in size but are still in complete spermatogenesis. Pigmentation and interstitial cells show little, if any, change since December 23. Tubules are smaller in diameter, however, and sperms are not so far matured as on that date.

Both mature and juvenile birds appear to have gone over the crest of spermatogenic activity, to different degrees, under daily light periods still lengthening. However, the mature birds have regressed much less than the juvenile ones.

g) *40-watt bulb.*—Testes of young birds (Plate III, Figs. 32, 33) show different degrees of regression. Some show vacuolated cytoplasm and necrotic nuclei, with Sertoli cell cytoplasm inclosing various stages of germ-cell nuclei (Plate III, Fig. 32), characteristic of early regressive stages in the natural cycle (Bissonnette, 1930*b*). Interstitial cells are again distinguishable in these testes, and pigment is closely aggregated. Testes of other birds (Plate III, Fig. 33) show conditions of regression reaching late autumn conditions, with great shrinkage of the tubules and loss of lumen. Nuclei within the tubules are reduced to the three sorts described above for midwinter and late autumn, in similar proportions. Pigment is dense and interstitials are numerous.

Testes of mature birds (Plate II, Fig. 14) are still near maximum size, with large tubules and almost complete spermatogenesis. Pigment and interstitials are in breeding stage. No signs of regression appear except reduced testis and tubule size.

h) *50-watt bulb.*—Testes of both young (Plate II, Fig. 21) and mature birds (Plate II, Fig. 14) are at maximum size and complete spermatogenesis with no signs of regression, but at the crest of breeding activity. Tubules in young birds are larger than in mature ones.

i) *60-watt bulb without added "work."*—Testes of mature birds are near maximum size and spermatogenic activity (Plate II, Fig. 21), but slightly smaller than those just described from the 50-watt cage. Conditions in the tubules do not differ essentially. They show slight advance since December 23. No young males were left for comparison.

No birds subjected to 60-watt bulbs and extra forced "work" were left to be sacrificed at this time. Comparisons of the reactions of birds with and without extra "work" but with added light periods per day terminated on December 23. All testes examined on that date from birds subjected to both added light and "work" were at complete spermatogenesis, with more or less matured sperms.

DISCUSSION

That some of the supposed hereditary inherent rhythms of biological reactions of animals and plants are due either directly or indirectly to environmental cycles, of which light periods and cyclic variations in light intensity are two factors, is becoming more and more evident as experimental accuracy increases. In some cases the rhythms disappear under carefully controlled conditions of environment.

Several studies on life-cycles in Protozoa indicate that rhythms of division rate and vitality depend on rhythms of light (Beers, 1928; Dawson, 1928; Richards, 1929). These studies fail to differentiate between the effects of daily period and changes in intensity. The starling studies show that for them, at least, change in intensity modifies reproductive cycles, as well as change in length of daily light period. Where daily period is constant, intensity of illumination determines the rate of acceleration of testis activity toward complete spermatogenic activity.

Many authors hold that the physiological effects of sunlight and artificial radiations are due in great measure to the luminous and infra-red portions of their spectra rather than to the 1 per cent of ultra-violet energy which reaches the earth's surface and is subject to altitude and seasonal and daily variations, and that the bad effects of tropical sunlight are due principally to absorption of heat rays and failure of cooling power. This, of course, is aside from the anti-rachitic effect confined to the ultra-violet portion. Even in the case of the latter, it is believed that the action of certain wave-lengths is increased by the simultaneous action of the visible and infra-red. This greater action of the longer visible and infra-red rays is believed to be due to greater penetration power, particularly of the visible rays. However, some parts of the spectrum affect some conditions more than others. More attention should be paid to wave-length, intensity, and energy content (Laurens, 1928). Our experimental results show effects from the visible portions of the spectrum and in almost total absence of ultra-violet, since all birds are housed behind ordinary window glass and the added light comes from ordinary incandescent bulbs.

It has been suggested that effects are due to the more deeply

penetrating visible wave-lengths which heat the subcutaneous tissue and the blood in it to greater temperatures than those of fever, without raising the general body temperature, which is kept down by body heat regulation. So some workers prefer carbon arcs to quartz-mercury lamps (Laurens, 1928; Sonne, 1926*b*[*1]). Our results do not bear on the mode of reception of light effects by these birds, but this may be the mode of primary effect. Direct penetration to the gonads and direct effects in these glands in starlings may be possible with intense enough light and long enough exposure.

That pigmentation protects against the radiant energy and that spring sunlight is relatively richer in the more penetrating visible and near infra-red rays than autumn and summer sunlight, while pigment is less well developed, while autumn and summer sunlight are stronger in ultra-violet and pigmentation is more dense, are well-known facts. This may account for the "spring fever" and frequent fatigue of spring caused by the deeper penetration of radiation from the visible and red end of the spectrum at that time in man (Laurens, 1928). It may possibly account also for the accelerating effects upon sexual activity in the starlings during that season, and the regression as ultra-violet becomes greater before June 15, as noted by Bissonnette (1930*b*); though the fact that several birds passed the crest of activity under our experimental conditions without the intervention of ultra-violet appears to indicate that the ultra-violet may be only a secondary factor at most. The pigmentation in the testes may protect from injurious effects of strong light when it is in the dense masses between the tubules in autumn, winter, and early spring, and permit a general acceleration of activity under visible light; but it may fail to protect when it is greatly scattered, as it is from April to June, by the great expansion of the unpigmented germinal elements of the testis (Bissonnette, 1930*a*, 1931). It is possible that the spring rush of activity in the tubules, resulting in dispersion and thinning-out of pigment, may leave the germ-cells so unprotected from the action of the deeply penetrating rays as to be injured by the cumulative action of the radiant energy received, and to undergo regression. This may account for the regression beginning before June 15 in testes stimulated to action by increasing daily daylight

[1] References marked with an asterisk are cited from Laurens' 1928 review.

periods, although daily daylight periods continue to lengthen till June 21 and intensity of illumination increases into July.

The question as to the origin of the pigment must be left till the pigment situation has had more study.

Stimulation of epithelization by visible and near infra-red rays has been shown to be the mode of action of light on wound-healing (Bernard, 1926*). In the starlings it is the germinal epithelium that is stimulated by light increases, whether it be directly by rays reaching it or indirectly by the mediation of some other organs or organ.

A relationship has been demonstrated between actual hours of sunshine, rate, extent, and persistence of the change in curve of sunshine, on the one hand, and the character of experimental disease, even when the rabbits used as subjects were behind glass, as were our birds. Definitely smaller and more stable organ weights have been shown to be correlated with constant electric light, except for the thymus, than in controls with normal lighting. Liver weight was greatly increased in total darkness, and other organ weights were smaller and more stable than in controls, but not so much so as in constant light. Constant light has also been found to induce lower malignancy in experimental disease than constant darkness, and both lower than controls with normal light periods. This is in accord with high malignancy during abrupt and rapid changes in hours of sunshine in spring and autumn and low malignancy in winter and summer (Pearce and Van Allen, 1926*, 1927*). Male starlings fail to undergo two rises and falls in activity of the germinal epithelium per year but show effects due to variations of light in either direction, rising to maximum in late April and May and falling to minimum in November and December (Bissonnette, 1930*a*, 1930*b;* Bissonnette and Chapnick, 1930). In this present study intensity is found to be a factor in the rate of change up to a definite intensity but not thereafter. Higher intensities above that from 60-watt bulbs at 9 inches distance were not tried. Up to 240 lumens at 9 inches distance from the center of the roost as emitted by a 25-watt bulb, and possibly to 408 lumens from a 40-watt bulb, at the same distance, with less intensity in other parts of the cages, there appears a correlation of degree of effect with light intensity. The 15- and 10-watt bulbs, with 131 and 71 lumens, respectively, at the

same distance from the roost, give progressively slower increases in activity of testes than those with 240 lumens and above. No constant differences in effects were produced by bulbs emitting light intensities from 408 to 678 lumens at the same distance. Forced exercise for the same periods with the added light of 678 lumens, at the same distance, prolonged the refractory period before light effects became manifest or delayed the onset of light-induced activity. Testes of some young birds passed the climax of spermatogenic activity and underwent various degrees of regression between 44 and 79 days of treatment, with increasing light up to the seventy-ninth day. Mature birds did not do so to so marked a degree, though one or two showed some signs of regression. None in cages subject to 15-, 50-, or 60-watt bulbs did so. This may point to a maximum state of activity maintainable only for variable short periods in some birds and longer periods in others, inevitably followed by regression even under conditions that otherwise would induce progressive changes. The length of this period of maximum activity may possibly be controlled by external conditions, but it was beyond control by changes or factors taken into account in our experiments to date. This does not preclude the possibility of its being conditioned by some factor or factors not tested in our experiments, such as much higher light intensity, longer periods, increased ultra-violet irradiation, or decreased relative amount of the longer red and infra-red rays in the summer spectrum.

In some ways the situation suggests a cumulative or summation effect of radiation such as that described by Grave (1920, 1926) and Mast (1921) as controlling the free-swimming period of certain tunicate tadpoles before attachment and metamorphosis.

Efficiency of reaction of rabbits to infection with syphilis increases with the amount of light and constancy of exposure to it, but changing light conditions may nullify the beneficial effects of light (Pearce and Brown, 1927*). Beneficial effects of radiation may be due to improved circulatory and metabolic conditions of the central nervous system (Hasselbalch, 1911*). Light may lead to beneficial growth and regeneration effects, especially in wound-healing by increasing cell-division rate (Hausmann, 1923*; Jesionek, 1912*). These effects

parallel the increased cell division of the germinal epithelium in starlings.

In *Drosophila* increased light usually interferes with the normal growth processes by causing the rate of aging to increase with the intensity of light (Northrop, 1925*, 1926*). Variations in effect occur above a threshold of light intensity of 2,500-meter candles for larvae and 1,000-meter candles for adults, for *Drosophila*. But our variations occur below a light intensity of 408 lumens at 9 inches distance. However, our tests did not go above 678 lumens at 9 inches, with these birds. Fluctuations, but no constant increase or decrease, occurred between 408 and 678 lumens. Nevertheless, we did find a difference in rate and degree of effect between juvenile and adult birds.

Direct irradiation of rabbits has been found to modify the relative weights of external parathyroids and hypophysis differently from that obtained by feeding cod-liver oil and irradiated cholesterol. There are, therefore, effects upon the animals not produced in the irradiated substances (Gates and Grant, 1926*, 1927*). Since the sex glands are correlated with the hypophysis, changes in the gonads of the starlings may possibly be mediated by the hypophysis. This has not yet been tested by experiment.

On the other hand, it has been found that cod-liver oil is more effective than either irradiated cholesterol or direct irradiation, on rats, in raising inorganic blood phosphorus in the prevention and cure of rickets (Koch and Cahan, 1926*). These effects are due to the ultra-violet and are therefore outside the regions of the spectral effects acting upon our birds. But long wave-length light with little energy and no ultra-violet can produce definite changes in the calcium and inorganic-phosphorus equilibrium in the blood of rabbits, and can modify growth, activity, nutrition, and hair growth (Brown, 1928). Blood equilibrium changes may possibly be concerned with changes in sexual activity of these birds.

While intense illumination does not change the basal metabolism in man, covering the eyes of grasshoppers decreases muscle tonus and reduces the rate of CO_2 output about 30–40 per cent so long as the eyes are blackened (Laurens, 1928; Bodine, 1922*). Our birds are active so long as lights are on and metabolism cannot come down to

basal level, but forced exercise without light did not lead to increased spermatogenic activity but rather to increased pigmentation (Bissonnette, 1930*a*, 1931). Forced exercise with added light leads to a lengthened refractory period before the appearance of light induced changes in the testes.

Although the real action of the radiations on living cells is yet unknown, we know that both stimulating and lethal effects are produced. Stimulating action results from wave-lengths longer than 290 m. μ; lethal action from the shorter wave-lengths. These effects arise from two component reactions—a photochemical action leading to dermatitis and its logical sequelae, and a biological action which may last for some time (Laurens, 1928). It is with this latter action that we are concerned in our work with the starlings.

Some believe that light energy absorbed by the skin sets up useful reflexes, is absorbed by the blood, stored, and carried to all parts of the body, stimulating the intra-cellular oxidation and reduction reactions; while others think that fresh air on the skin is responsible for the increased tone and well-being in man (Laurens, 1928; Rollier and Rosselet, 1925*). It is generally believed that most reactions to radiation are oxidation-reduction processes, involving valency changes, and that the effect of short-waved radiation is due to reduction of certain plasma compounds. Many also hold that "visible heat rays" from arc lamps heat a very essential fraction of the blood-volume of an animal to a temperature possibly exceeding the highest measured fever temperatures and that the specific heat of this blood, carried throughout the body, produces beneficial effects (Laurens, 1928). It is possible that this is the case in the starlings up to a certain optimum, while higher temperatures, so produced, may be deleterious to gonad function and cause regression during daily illumination periods of greater length. This is in accord with the noted onset of regression in those birds that passed the crest of spermatogenesis under constantly increasing daily light periods in these experiments, as well as normal regression before June 15 (Bissonnette, 1930*b*). However, it would not be necessary in order to explain regression resulting from shortened light periods before the crest of activity is reached in some birds, while birds receiving the same daily illumination continue to progress, if their previous daily light periods have been shorter (Bissonnette, 1931). In that case,

not total irradiation, but relative, conditions the direction of change in spermatogenic activity.

In man, pigmentation protects against the short-waved ultra-violet, and aids in penetration and absorption of light energy as heat, by lessening reflection; and so the effects of illumination are heat effects (Laurens, 1928). If, as is likely, these situations apply in the starlings, pigmentation of the intertubular regions of the testes may act as protection to the germinal epithelium against harmful radiations, and, when added to the pigment of the feathers of the back of the bird, constitute an effective protection under moderate conditions of light. This may fail to give full protection under long-continued or under lights rich in ultra-violet. It may contribute to the local warming of the tissues without dangerous rise of general body temperature. This local heating effect may become too great in summer or under artificial conditions and cause the regression of testis activity, mentioned above.

Another suggestion is that radiation shifts the mineral balance, and sets free, into the blood, protein decomposition products leading to endocrine and autonomic system changes which set up chemical reactions in remote parts by products of damaged or activated cells or activated substances in blood or lymph (Kroetz, 1926*). Parathyroids are sensitive to sunlight on the skin and react by hypertrophy. Absorption and excretion of calcium and phosphorus by the bowel is also affected. These changes are not incompatible with the findings of this study. Chemical changes in the skin may be forerunners of substances effective elsewhere—in the testes.

One conception of the mode of action of all radiant energy on protoplasm is that it increases molecular motion with lethal effects when direct atomic disintegration occurs in molecules. The effect of any given wave-length is directly proportional to the coefficient of absorption of the given protoplasm for that wave-length. While all radiation transfers energy to molecules as heat, some wave-lengths break up molecules when the absorbed energy reaches a certain point. Which rays do this depends on the characters of the molecules reached by the rays. This view attributes the activities of vitamines to their excess energy, derived from the ultra-violet or other sources, which they can liberate to aid basic chemical body-processes (Laurens, 1928). It is the physiological condition of the

organism or organ that determines the nature of the response, since rays from widely separated regions of the spectrum, if they penetrate to the same parts, give similar physiological effects regardless of difference in wave-length, and differences of effect become due to differences in penetrating power rather than to action, specific for wave-length (Bovie, 1925*). This is in accord with the greater effect of stronger bulbs in our experiments on the starlings. However, greater effects occur only up to intensities given by 40-watt bulbs, and not above. The question as to the possibility of different effects or degree of effect of different regions of the spectrum is being tested and will be reported upon later.

It has been suggested that these effects are photochemical and due to changes in configuration and velocity of electrons of the substance absorbing radiant energy, which, if the incident energy has short enough wave-lengths, produce electron vibrations instead of molecular or atomic ones and expel electrons from the molecules, ionizing them, or displace the electrons to an outer orbit, thereby activating the atoms or molecules, and cause altered chemical reactions in the absorbing substances, often with the aid of sensitizers (Clark, 1924*b**; Laurens, 1928; Saidman, 1925*; Koenigsfield, 1921*; Hill, 1924*a**, 1924*b**, 1926*). Such photochemical changes occur in the skin or organs reached, and products pass to affect other organs. Lipoid changes in the germinal epithelium of the crow, followed by the appearance of similar changes in surrounding tissue, such as interstitial cells during regression testis changes, have been observed and suggested as possible sources of the testis hormone in birds (Orban, 1929). Similar changes appear in the testes of the starling, affecting fibroblastic cells.

Rapid hydrotropic or coagulative degenerative changes have been noted in white blood cells of various mammals in hanging drop cultures when irradiated with various commercial incandescent bulbs with wave-lengths distributed between 430 and 600 m μ. Erythrocytes under aërobic conditions undergo swelling and hemolysis under similar treatment, but not in anaërobic conditions. Fibroblasts from 6- to 12-day chick embryos, in the presence of erythrocytes but not otherwise, undergo degeneration leading to death (Earle, 1928*a*, 1928*b*, 1928*c*). These findings are suggestive as to the mode of action of light, either directly or indirectly through degeneration products, upon the testes.

In regard to the mode of action of increased muscular work, in prolonging the refractory period before light-induced effects appear in our experiments, we are not yet prepared to theorize. In this connection, however, the literature gives evidence for enhanced protein metabolism resulting from increased muscular activity, though most of the energy involved comes from fats and carbohydrates (Cathcart, 1925). It is possible that waste products from such protein metabolism may cause the delay mentioned above.

Changes in light intensity have effects on refractory periods in the eyes of *Eristalis tenax* (Dolley, 1930) and on color changes in the leech *Protoclepsis tesselata* Broun (Stchegolew, 1927).

Ceni (1922), as the result of experiments in blinding cocks, concluded that vision exerts an effect upon the reproductive glands through the higher nervous centers controlling reproduction, though, as Dalcq (1923), in abstracting his paper, points out, the conclusion is not entirely warranted by the observed facts. The results do suggest the possibility that sight may be one of the avenues through which light affects the reproductive mechanism in addition to the skin. This possibility may be tested in future experiments with the starlings both as to psychical and physico-chemical effects.

Cases of lunar cycles in relation to May-fly swarms (Hora, 1927) and to reproductive cycles among worms, such as *Nereis*, and the palolo worm or epitoke of *Leodice viridis* in Samoa and Fiji or *L. fucata* in the Atlantic, and other animals are well known. These reproductive cycles may well be conditioned by extra intensity of added light after sundown due to moonlight, resembling in effects the added electric light of our experiments and correlated with the changes in duration and intensity by the moon's phases.

That cosmic excitants of unknown nature, most abundant in spring and autumn, correlated with variations in muscle glycogen and with changes in the sex-gland activity, and with the condition of organisms as wholes, possibly come from the sun and stars and account for observed seasonal changes of physiological activity has been postulated (Maignon, 1921). Possibly these are of the same nature as the light causing the observed changes in starling testes.

That light-intensity variations are operative among plants as well as animals is well illustrated by the appearance of green plants (protococcales, liverworts, fern prothallia, and ferns) in caves when

100-c-p. lamps replaced 60-c-p. ones (Matthews and Hamilton, 1928).

Daily light periods are effective in conditioning sexual activity with Aikoku rice (Noguti, 1927). However, longer periods inhibit flowering in these plants, while shorter ones accelerate it. This is the reverse of the effect in starlings. Sexual reversal in hemp is induced by shortening daily daylight periods, roughly in inverse ratio to the length of daily daylight period, while relatively long daily periods condition pure sexual expression as male or female without sex reversal (Schaffner, 1923).

Starlings do not undergo sex reversal when daily light periods are shortened, but revert to the infantile state of the germinal epithelium in both sexes. Increased light leads to extreme activity and maturity in either sex.

There is a difference in rapidity and limits of response between juvenile birds of the previous summer's broods and mature birds, which have been sexually mature at least once before. Mature birds, on the whole, respond more quickly and to a greater degree than do juveniles, under the same conditions of light and work.

SUMMARY AND CONCLUSIONS

1. Six groups, of 12 birds each, were subjected to light from 10-, 15-, 25-, 40-, 50-, and 60-watt incandescent bulbs without forced exercise and 11 birds to light from 60-watt bulbs with forced exercise, for equal periods nightly, rising by from 15 to 20 minutes per night up to 6 hours and thereafter kept constant until the end of the experiment.

2. These electric light periods were added to a normally decreasing day-length from November 9 until December 21, and to a normally increasing day-length thereafter until January 27, in a basement room lighted by day through three large windows, and kept cool by having the windows open for a few inches at the bottom.

3. A control group of 12 birds received neither added direct light nor work after sundown.

4. Rations of all groups were in excess of their needs during the day. Food was continuously before them all during daytime, and before all except the 60-watt-treated cages at night.

5. No deaths occurred from any cause during the 79 days from November 9 to January 27.

6. Birds were killed for gonad study on December 13, 23, and January 27 at 34, 44, and 79 days, respectively; and a few were carried over into the experiments on effects of lights of different wave-lengths, which followed, beginning January 28 with a new set of birds. Outside controls were killed for comparison at 79 days on January 28.

7. Study was made by direct microscopic studies of testis sections, and by comparison of photomicrographs of comparable regions of all testes, made to the same magnification (335 diameters), and $3\frac{1}{4}\times4\frac{1}{4}$ inches in size. This latter procedure makes comparisons very accurate as to tubule size, relative number of germ-cell elements, interstitial cells, and pigment masses.

8. A method of distinguishing the sexes, in winter and juvenile plumages, is described.

9. Conditions of all testes studied are described in detail as to size of testis, tubule diameter, pigmentation, germ-cell layers and activity, and relative progression and regression conditions.

10. All the electric-light intensities used caused progressive testis changes and increased germ-cell activity with the daily periods used, reaching complete spermatogenesis in 44 days, but not at the same rate. Some juvenile birds, from cages receiving 10-, 25-, and 40-watt light, passed the climax of spermatogenesis and regressed to various degrees before 79 days of treatment, even with the normally increasing daylight periods from December 21 to January 27.

11. Rate of acceleration of spermatogenic activity increased with intensity of light from 10-, 15-, 25-, and possibly to 40-watt bulbs, but for 40-, 50-, and 60-watt intensities there was no constant difference shown at either 34 days or later. There may have been detectable differences before 34 days however, but no birds were killed and studied earlier. It is possible that the higher intensities show appreciable differences in very early rates of acceleration and that all our birds had reached a relatively long slow stage of progression before any were killed and examined.

12. Light intensities from the bulbs used, as given in lumens by the firms selling us the bulbs, are:

10-watt bulb= 71 lumens	15-watt bulb=131 lumens
25-watt bulb=240 lumens	40-watt bulb=408 lumens
50-watt bulb=525 lumens	60-watt bulb=678 lumens

and 1 lumen is equal to $\frac{1}{4\pi}$ candle intensities from a uniform point source.

13. There is a difference in rate and degree of response between juvenile birds from the previous summer's broods and mature birds which have been at sexual maturity one or more times previously. Testes of juvenile birds give neither as rapid nor as great increases in testis size and germ-cell activity as do mature birds, under like conditions of light and work.

14. In the cases observed, juvenile birds were found to be more susceptible to the action of regression-inducing conditions than mature birds, but not all juvenile birds underwent regression in the time covered by these studies.

15. Data suggest a refractory period between the initiation of light changes and the appearance of light-induced effects, prolonged by added muscular exercise, together with individual and age differences in susceptibility to progression- and regression-inducing stimuli.

16. Added exercise, in the case of 60-watt-treated birds, caused a lag of onset of the light induced changes, both progressive and regressive.

17. Total testis size, produced in midwinter by added electric light, equals, and may even surpass, that produced in spring during the normal sexual cycle, as shown by some cases here described.

18. A discussion of the literature on biological effects of light, from the visible and infra-red portions of the spectrum and from electric-light bulbs, upon both animals and plants and of possible modes of action is given.

19. Intensity of illumination, as well as duration of daily light period, is a factor in conditioning the degree of testis activity and testis size in the sexual cycle of males of the European starling (*Sturnus vulgaris*); and, for this, electric light may replace sunlight. Age is a factor of minor importance.

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THE ACTION OF ULTRA-VIOLET RADIATION ON LIMAX FLAVUS LINNAEUS. I. THE VARYING EFFECTS ON NON-PIGMENTED AND PIGMENTED EMBRYOS

EMMETT B. CARMICHAEL
Department of Physiological Chemistry, School of Medicine,
University of Alabama

THE present paper deals with the effects produced by ultra-violet rays on the eggs containing living embryos of the nocturnal slug, *Limax flavus* L.

Hasselbalch (1911) performed experiments in which he demonstrated that the skin transmits very little of the ultra-violet rays shorter than 2,900 A except where the epidermis is less than 0.1 mm. thick. Macht and co-workers (1925) noted that with the skin of the negro absorption of the entire ultra-violet region occurred. Peacock (1925) reported that ultra-violet rays were absorbed by both melanin and sebum. Clark (1922) believed that pigment insured a maximum absorption of radiant energy in the basal cells of the epidermis, and that it therefore might result in an increased light reaction in that part of the body.

The action of ultra-violet rays on *Arbacia* eggs and on starfish eggs was studied by Lillie and Baskervill (1922*a*, 1922*b*). They concluded that the radiation produced primarily a surface effect.

Burge (1916) thought that ultra-violet rays injured or killed living cells by coagulating or rendering insoluble the protoplasm or living material of the cells.

Ergosterol has been transformed or converted into vitamin D, the vitamin that protects against rickets, by ultra-violet rays (Rosenheim and Webster, 1927; Hess and Windaus, 1927). Massive doses of irradiated ergosterol which were fed to young rats on a ricketogenic diet caused them to lose weight (Klein, 1929; Kreitmair and Moll, 1928; Harris and Moore, 1928).

The slug eggs which were used in the present paper for testing the action of ultra-violet rays were divided into three groups: (1) embryos that had pigment in their mantles as well as in their eyes,

and thus were almost ready to hatch (19–23 days after they were laid); (2) large embryos with pigment in their eyes (14–18 days after they were laid); and (3) embryos that had no pigment but which were definitely forming (7–13 days after they were laid). The time required for hatching was found to vary from 19 to 27 days when the eggs were kept at room temperature, and the majority of the batches of eggs started to hatch by the twenty-third day.

TABLE I

EGGS CONTAINING LIVING EMBRYOS OF *Limax flavus* L. TREATED FROM 5 TO 20 MINUTES WITH ULTRA-VIOLET RAYS

Size of Embryos in the Eggs	No. of Eggs Exposed	Length of Exposure (Min.)	Total No. of Eggs to Hatch	Total No. of Young That Lived
Large embryos. Pigment in eyes and mantles	19	5	19	3
Large embryos. Pigment in eyes	38	5	17	16
Embryos definitely forming. No pigment	34	5	30	30
Large embryos. Pigment in eyes and mantles	19	10	13	None
Large embryos. Pigment in eyes	37	10	5	None
Embryos definitely forming. No pigment	34	10	7	6
Large embryos. Pigment in eyes and mantles	40	15	14	None
Large embryos. Pigment in eyes	46	15	None	None
Embryos definitely forming. No pigment	24	15	None	None
Large embryos. Pigment in eyes and mantles	40	20	2	None
Large embryos. Pigment in eyes	36	20	None	None
Embryos definitely forming. No pigment	13	20	None	None
Large embryos. Pigment in eyes and mantles	325	Controls	319	319
Large embryos. Pigment in eyes	42	Controls	42	42
Embryos definitely forming. No pigment	39	Controls	35	35

As a source of light, a Burdick air-cooled quartz mercury vapor lamp was used on a 110-volt A.C. circuit. The lamp was turned on 15 minutes before exposures were started, in order to insure a constant source of ultra-violet rays. At the end of this period, the voltmeter always registered about 65 volts. The eggs, which were in open Petri dishes on moist filter paper, were placed 30 inches beneath the center of the quartz tube.

After being separated into three groups, as explained above, the embryos were treated for different periods of time, using all the rays from the lamp. The results are shown in Table I.

Some of the embryos, Group 1, hatched after a continuous exposure of 15–20 minutes to all the rays from the lamp, but these embryos always died within 2–7 days after hatching. Even with

treatments of 5–10 minutes, the embryos were markedly affected, and most of them died within a few days. Those that survived had swollen mantles, and the anterior portions of their mantles were wrinkled and elevated. The embryos of Group 2 withstood the treatment much better than the embryos which had pigment in their mantles and in their eyes. The embryos of Group 3 seemed to be less affected by the rays, as all that hatched, after 5 minutes' exposure, lived. Several young embryos of group 3 lived after 10 minutes' exposure to all the rays from the lamp.

TABLE II

EGGS CONTAINING LIVING EMBRYOS OF *Limax flavus* L. TREATED FROM 3 TO 7.5 MINUTES WITH ULTRA-VIOLET RAYS

Size of Embryos in the Eggs	No. of Eggs Exposed	Length of Exposure (Min.)	Total No. of Eggs to Hatch	Total No. of Young That Lived
Large embryos. Pigment in eyes and mantles	30	3	30	30
Large embryos. Pigment in eyes	18	3	17	16
Embryos definitely forming. No pigment	55	3	47	47
Large embryos. Pigment in eyes and mantles	18	4	17	8
Large embryos. Pigment in eyes	13	4	11	9
Embryos definitely forming. No pigment	18	4	16	16
Large embryos. Pigment in eyes and mantles	36	5	34	9
Large embryos. Pigment in eyes	25	5	21	20
Embryos definitely forming. No pigment	57	5	50	50
Large embryos. Pigment in eyes and mantles	20	6	19	None
Large embryos. Pigment in eyes	14	6	13	11
Embryos definitely forming. No pigment	17	6	16	14
Large embryos. Pigment in eyes and mantles	22	7.5	18	None
Large embryos. Pigment in eyes	25	7.5	16	16
Embryos definitely forming. No pigment	45	7.5	33	33
Large embryos. Pigment in eyes and mantles	33	Controls	34*	34
Large embryos. Pigment in eyes	23	Controls	23	22
Embryos definitely forming. No pigment	50	Controls	46	46

* One of the control eggs contained twins.

The embryos used in the next series of experiments were selected according to their size and degree of pigmentation, the same as those were in the previous experiments, Table I. The lengths of the exposures were reduced to less than half of those in the former experiments, and the range of the treatments was also reduced. The results are included in Table II. From the results of this table it can be seen that the embryos which had pigment in their mantles and in their eyes were more severely affected by ultra-violet rays

than either those which had pigment only in their eyes or those which had no pigment at all.

The following control experiments were performed in support of the view that the effects of the treatment with all of the rays from the quartz mercury lamp are due to the rays of short wave-length:

a) Since the lamp raised the temperature of the surrounding atmosphere, embryos of each group were placed in an incubator at 32° C. and kept there from 1 to 48 hours. This temperature was from 2° to 6° C. higher than that of the surrounding atmosphere during the treatment of any embryos included in Tables I and II. The embryos of Groups 2 and 3 were more sensitive to the increased temperatures than those of Group 1. The embryos of Group 1 lived and hatched after 20–24 hours' incubation.

TABLE III

Filter No.	Salt	Concentration	A
1.....	Ammonium sulphate	Saturated	2,030
2.....	Lead acetate	1–400	2,540
3.....	Copper sulphate	1–40	2,880

b) Glass color filters (corex), which transmit the ultra-violet rays, were employed; and the embryos of Group 1 were severely affected if treated from 15 to 20 minutes. The embryos of this group were not affected by a 20-minute treatment under a clear filter (corex), which absorbs 2,800 A and shorter. Window glass (2.2 mm. thick) was effective in protecting the embryos of Group 1 for 1 hour, and a Petri dish cover (1.4 mm. thick) protected them for 20 minutes.

c) Aqueous solutions of ammonium sulphate, lead acetate, and copper sulphate were used as filters. Their corresponding values in wave-lengths are given in Table III (Schneider and Sperti, 1926; Carmichael, 1929).

Filter No. 3 protected embryos of Group 1 for 10 minutes, while the other two filters did not.

The quartz vessel used to hold the aqueous filters was charged with the same amount of distilled water, and embryos of each group were treated 5, 7.5, and 10 minutes. Again the embryos of Group 1 were the only ones to have swollen and wrinkled mantles, which

are characteristic effects produced when these embryos are treated with all the rays from the lamp. This experiment also shows that ozone was not responsible for the deleterious effects produced by the direct radiation.

DISCUSSION

Since the embryos of Group 1 which were treated several minutes with all the rays from the lamp hatched and in many cases lived a few days, it seems probable that death was not due directly to the rays. The embryos which died within a few days after hatching received what has been termed a "lethal dose" of the ultra-violet rays. The pigmented embryos which received a sublethal dose often showed marked injurious effects, which were visible for 2 or 3 weeks, and then the young slugs appeared to be restored to normal. It seems probable, as Baldwin (1921) has pointed out in the case of tadpoles, that the organism rectifies itself by means of a delayed process of chemical postgeneration.

Schneider and Sperti (1926) think that it is probable that the destructive action of radiations on living things is due to the dissociation or decomposition of chemical compounds. The effects produced on the embryos might be due to either or both of the following causes: (1) the absorption of the ultra-violet rays by the pigment and the conversion of these rays into heat, and (2) the action of the ultra-violet rays on certain of the protoplasmic substances—for example, such as the sterols. According to the works of other investigators on the action of light on pigment, it seems that the deeply pigmented embryos would absorb more of the ultra-violet rays than the embryos which had pigment only in their eyes or which had no pigment. Since the majority of the older embryos which were deeply pigmented died if exposed for 5 minutes or longer to all the rays from the quartz mercury vapor lamp, there seems to have been in these young slugs a greater light reaction or chemical action than in the slugs of the other groups.

SUMMARY

1. Pigmented embryos of the nocturnal slug, *Limax flavus* L., are easily injured by ultra-violet rays.

2. The deeply pigmented embryos are especially sensitive to ultra-violet rays, a few minutes radiation being lethal for them.

3. Non-pigmented younger embryos are not so easily injured by ultra-violet rays as are the older pigmented embryos.

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AVAILABLE FOOD AND CROWDING AS FACTORS INFLUENCING THE SEX OF MOINA MACROCOPA. I

(One figure)

C. A. STUART, JUANITA TALLMAN, AND H. J. COOPER
Brown University

IN A previous paper by Stuart and Banta (1931) it was shown that under certain experimental conditions a definite ratio existed between the number of males produced by crowded *Moina* mothers and the number of viable bacteria present in the medium in which the mothers were reared. In these experiments crowded *Moina* mothers were cultured in clear, paper filtrates of dilute manure infusions bacterized with varying amounts of a suspension of *Aërobacter aërogenes*. The number of males produced by these mothers paralleled very closely the number of viable bacteria present in the various culture bottles. An adequate number of bacteria being present, approximately one billion per milliliter, mothers produced only female young. As the number of bacteria was decreased in a series of bottles, males were produced, the percentage of males being in direct proportion to the decrease in the bacterial count of the medium. In several experiments the numbers of bacteria in the filtrate were reduced to the point where *Moina* mothers produced 100 per cent males. Decreasing the numbers of bacteria beyond this point, however, resulted in the reappearance of female offspring from the mothers crowded in these bottles.

It was also found that the total number of young released by the crowded mothers was roughly proportional to the number of bacteria present in the medium and to the number of males produced. The maximum number of young was produced, not in the bottles containing the maximum number of bacteria, but in the bottles containing fewer bacteria wherein the sex ratio was approximately three females to one male. From this point the number of total young decreased steadily as the bacteria decreased, although the number of males continued to increase until the

higher dilutions were reached. Before these experiments were completed, Stuart, McPherson, and Cooper (1931) showed, by rearing bacteriologically sterile *Moina* in sterilized normal medium bacterized with pure cultures of *Aërobacter aërogenes*, that the food value of this species of bacteria was equal or superior to that of unbacterized normal medium. It was, therefore, tentatively concluded that the factor controlling the production of male or female young was the number of viable bacteria present as available food in the medium.

If the conclusion that food is the controlling factor in sex production is correct, then similar results should be obtained when crowded mothers are reared in various dilutions of normal medium, since Stuart and Banta (1931) have shown that with sufficient dilution the food value of such a medium is proportional to the dilution. Banta and Brown (1929*a*, 1929*b*), however, present evidence to the contrary, inasmuch as they found that rearing mothers in dilute food (dilutions of normal medium) or in clear pond water, even with crowding, usually failed to cause male production or gave a reduced percentage of males. No experiments are reported on this phase of their work, and it is not improbable that these investigators worked only with extreme dilutions of normal medium, in which case one should obtain a reduced percentage of males or no males at all. It seemed advisable, then, to study the effect of rearing crowded mothers in varying dilutions of normal medium, since such an environment more closely approximates that in which the Cladocera are found in nature than does the bacterized filtrate used by Stuart and Banta (1931).

MATERIALS AND METHODS

Two liters of pond water were added to 700 grams of dry garden soil and 120 grams of horse manure (dried at room temperature for 12–24 hours). The mixture was allowed to infuse at room temperature for about 48 hours. At the end of this time any manure floating on the surface was carefully removed and the supernatant fluid was strained through 13XXX bolting-cloth. A small handful of the soil from the bottom of the jar was then placed in the cloth with the last 150 ml. of supernatant fluid and stirred with a glass rod in

order to supply the medium with silt. This filtrate constituted the stock solution from which all dilutions were prepared (Banta, 1921).

After several preliminary experiments to ascertain the dilutions that might best be employed for the work, the following series of dilutions was selected: 1:1, 1:2, 1:3, 1:5, 1:7.5, 1:10, 1:12.5, 1:15, 1:20, 1:25, 1:50, 1:100, 1:150, and 1:200. Pond water was used in making all the dilutions of the stock solution, and all experimental bottles contained 60 ml. of the specified dilution. Ten young (first instar) female *Moina* were placed in each bottle and the series incubated at 24°–26° C. Throughout all work reported in this and previous papers, young *Moina* were washed in at least one change of pond water before they were placed in the experimental bottles. In all the preliminary experiments and some of the first experiments reported in this paper, 10 sister-young were placed in each bottle, all the young in any one experiment coming from sister-mothers. Before the work had progressed very far, however, it was noted that occasionally one or two bottles in a series gave a sex ratio quite different from the preceding or subsequent bottle. It was considered that in such a bottle the Cladocera were at fault rather than the medium, since in carrying stock cultures of these animals it has often been noted that the young from a certain mother may be of one sex while the young from a sister-mother may be of the opposite sex. Such variation from the normal behavior of *Moina macrocopa* will be discussed at greater length in a subsequent paper. Granting, for the present, the possibility of variation in the young of sister-mothers used in any one experiment, it seemed more advisable to have the results of such variation distributed throughout all the bottles of a series rather than confined to a single bottle. Accordingly, 14 young, the progeny of a single mother, were distributed one to each dilution in a series. This was repeated with nine other clutches of young. Experiments set up in this manner appeared to give more consistent results.

In any experiment, as soon as the mothers laid eggs in the brood chamber, the mothers in dilutions 1:1 through 1:5, and occasionally 1:7.5, were transferred to normal medium (dilution about 1:12), since, as previously reported, Cladocera remaining in the higher concentrations of the infusion until the time of release often died in

greater numbers, or, should they survive to release their broods, the correct counting and sexing of the young were practically impossible due to the dense turbidity and color of the medium in these bottles. The mothers can be changed to a more suitable environment without influencing the results of the experiment, because the sex and the number of eggs have been determined (Banta and Brown, 1929*c*) before the transfer is made. In view of the fact that the bacterial count in dilutions 1:1 through 1:5 is changed very little (Stuart and Banta, 1931) during the course of the experiment, it was considered imperative to use these dilutions to observe the effect of a nearly constant quantity of bacteria on the sex ratio of the young produced by *Moina* mothers reared in these dilutions.

When the mothers released, the total number of young and the sex of at least 100 young from each bottle were determined. Whenever the total number of young in any bottle was less than 100, the sex of all the young was usually determined. It should be mentioned here that one finds it extremely difficult to determine the sex of some of the young released in the higher dilutions of medium (1:50–1:200). In some experiments animals were found in which one antenna was long, angulated, and blunt, as is characteristic of the male, but the other, though blunt, was short and non-angulated, as in the female. Animals have also been found with two short, straight antennae; and, if not observed closely, these animals might very easily be mistaken for females, but have been classed as males because the antennae have blunt rather than rounded ends. These facts admit of possible errors in sexing which should be taken into account. We are inclined to think that these peculiarities represent deformations of structures rather than sex intergrades, though the latter conception is by no means impossible. Males have been found with but a single antenna, with one or both antennae bent back over the head, and with antennae possessing distorting twists and curves. We have noted no deformed structures in any female young released in these experiments.

In some of the later experiments it was felt that considerable information might be gained not only from the sex and total number of young but also from the number of eggs present in the brood chamber for second release (hereafter to be called "second eggs"), since

TABLE I

| Series No. of Exp. | Date of Exp., 1930 | Dilutions of Normal Medium | | |

TABLE I

Dilutions of Normal Medium

1:5				1:7.5				1:10				1:12.5				1:15				1:20			
Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males
...	102	89	13	...	...	130	75	49	...	...	169	32	72	...	...	116	77	25	...	...	102	77	25	...	...	103	21	82
...	143	79	14	...	...	149	62	42	...	...	203	25	75	...	...	159	49	56	...	...	116	66	50	...	...	112	42	60
...	135	126	9	...	...	109	109	0	...	...	130	91	39	...	...	132	63	69	...	...	109	56	53	...	...	111	45	66
...	123	123	0	...	...	127	106	21	...	...	135	103	32	...	...	118	58	50	...	...	98	51	47	...	...	91	54	37
...	149	108	0	...	...	128	104	0	...	...	203	125	12	...	...	169	138	0	...	...	197	123	0	...	...	180	101	14
...	155	120	0	...	...	149	130	0	...	...	195	129	0	...	...	152	81	19	...	...	158	94	18	...	...	114	86	26
...	117	41	76	...	...	142	65	51	...	...	109	88	11	...	...	105	64	41	...	...	130	29	72	...	...	148	54	46
...	76	51	25	...	...	99	28	71	...	...	115	49	54	...	...	144	4	96	...	...	120	68	32	...	...	127	56	44
...	168	131	0	...	...	182	122	32	...	...	157	86	14	...	...	135	61	74	...	...	148	17	84	...	...	121	40	81
...	154	99	0	...	...	187	157	9	...	...	145	96	49	...	...	142	72	44	...	...	120	91	19	...	...	142	54	32
18	126	105	0	10	10	129	112	0	8	10	79	79	0	11	16	150	94	9	6	12	145	92	23	11	10	142	89	24
18	133	107	0	8	18	128	105	0	10	18	109	103	6	10	14	140	97	16	10	14	136	91	15	10	10	128	93	24
12	124	124	0	10	14	128	112	4	10	14	125	92	11	10	10	101	79	20	6	10	103	62	41	10	4	115	62	53
14	120	120	0	9	12	94	94	0	10	12	95	80	15	10	8	99	76	23	10	10	108	65	43	10	4	91	42	49
24	64	64	0	10	16	102	86	16	10	16	126	126	0	9	16	84	84	0	9	14	150	85	14	10	8	116	91	25
45				47				48				50				41				51			
16.57				13.87				14.16				12.80				12.14				7.25			
1,889				1,983				2,095				1,946				1,940				1,841			
1,487				1,467				1,304				1,097				1,067				930			
137				295				390				542				536				663			
8.43				16.74				23.02				33.06				33.43				41.61			
12.60				12.36				11.12				11.48				15.65				11.60			

1:25				1:50				1:100				1:150				1:200			
Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males
...	...	89	64	25	...	...	95	6	69	...	...	77	11	46	...	...	35	34	1
...	...	67	14	53	...	...	102	7	43	...	...	67	20	33	...	...	38	27	11
...	...	83	66	17	...	...	96	6	44	...	...	62	6	44	...	...	36	18	18
...	...	81	61	20	...	...	94	9	54	...	...	65	27	38	...	...	49	44	5
...	...	171	72	28	...	...	99	10	49	...	...	59	13	46	...	...	43	41	2
...	...	172	91	1	...	...	111	11	51	...	...	55	49	6	...	...	49	49	0
...	...	104	57	47	...	...	109	0	63	...	...	70	55	0	...	...	42	35	7
...	...	91	38	13	...	...	136	13	65	...	...	99	43	20	...	...	41	28	13
...	...	146	28	81	...	...	71	43	28	...	...	68	28	40	...	...	76	70	6
...	...	141	47	57	...	...	96	45	51	...	...	57	50	0	...	...	39	39	0
11	10	120	86	34	10	8	68	27	41	10	8	54	15	39	...	...	45	38	7
8	8	87	65	22	9	10	87	34	53	9	10	48	12	36	...	...	41	37	4
10	2	112	38	74	10	0	84	18	66	10	0	49	38	11	9	0	28	23	5
9	0	93	40	53	10	0	43	8	35	10	0	35	25	10	9	0	23	20	3
7	8	71	38	33	9	4	46	13	33	10	0	84	48	15	8	0	72	22	28
45					48					49					26				
5.55					4.29					3.46					0				
1,628					1,337					949					657				
805					250					440					525				
558					745					384					110				
40.93					74.87					46.60					17.32				
10.73					6.83					5.51					4.73				

1:200					
Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	
...	...	56	56	0	
...	...	69	44	8	
...	...	50	42	8	
...	...	62	44	6	
...	...	62	46	6	
...	...	29	22	7	
...	...	20	14	6	
...	...	46	40	6	
...	...	23	14	9	
...	...	27	17	10	
8	4	22	12	10	
10	6	41	18	23	
No Releases					
No Releases					
3	0	9	8	1	
21					
4.38					
516					
377					
100					
20.96					
3.42					

their number might reflect the environment in which the mothers were reared even more accurately than the number of young in the first brood. It was considered that the number of second eggs from

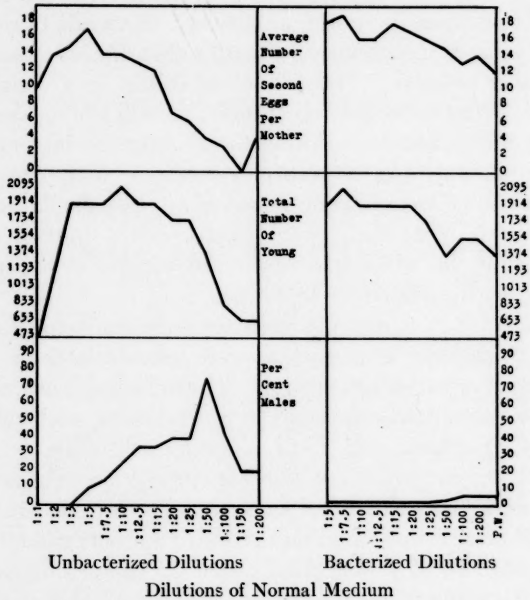

FIG. 1.—Graphic presentation of the results obtained from the experiments tabulated in Tables I and II. The different curves show the relationship between the number of second eggs per mother, the total number of young, and the percentage of males obtained from ten mothers reared in 60 ml. of various dilutions (ordinates left) of normal medium, and from ten mothers reared in 60 ml. of similar dilutions (ordinates right) of normal medium bacterized. It will be noted that dilutions 1:1, 1:2, 1:3, and 1:150 are omitted and that P. W. (pond water) is added in the bacterized dilutions.

those mothers which were transferred from a concentrated medium to one of greater dilution would be a reflection of the second rather than the first environment, that is, the more dilute rather than the highly concentrated medium. This assumption was by no means wholly correct, as will be shown later. The results of these experiments are tabulated in Table I and are graphically shown in Figure 1.

Before discussing in detail the results of this experiment, a second,

somewhat similar, experiment will be described in order that the two may be discussed together. Having obtained from the first experiment a sex ratio very similar to that obtained with the bacterized filtrates, in that the percentage of males increased as the dilution of the normal medium increased, an attempt was made to restore the nutritive value of the various dilutions by the addition of suspensions of *Aërobacter aërogenes*. This procedure should, in a certain sense, serve as a control to the first experiment, since in adding bacteria one is, within limits, merely restoring to the dilutions in concentrated form the original food value of the medium. Assuming that the various dilutions are adequately bacterized, one should obtain, for the most part, only female young from crowded mothers reared in the same dilutions, which when unbacterized, give male percentages dependent on the degree of the dilution.

Dilutions 1:1, 1:2, and 1:3 were not included in this second experiment, since only female young were released in these dilutions of the normal unbacterized medium. The following dilutions, 60 ml. to a bottle, were prepared from the concentrated stock solution as previously described: 1:5, 1:7.5, 1:10, 1:12.5, 1:15, 1:20, 1:25, 1:50, 1:100, 1:200; and in addition a bottle of pond water was added to this series. Untreated pond water was not included in the first experiment after it was found that crowded mothers seldom released in the average lot of normal pond water. In bacterizing these dilutions, no attempt was made to restore the original bacterial count of the medium before dilution. Instead, arbitrary amounts were taken, and the method of trial and error employed until satisfactory results were obtained. To exactly duplicate the original count of the medium would be impossible, since at least 48 hours would be required for a viable count on the medium and dilutions in question, and at the end of this time the count obtained would be no indication of the numbers of bacteria to be added to the dilutions since further changes in the bacterial flora of the medium would have occurred. In the first few experiments reported in Table II, about 0.4 ml. (depending to some extent on the nature of the medium) of the usual suspension of *A. aërogenes* (the growth of a 48-hour agar slant culture suspended in 3 ml. of pond water) was added to the first dilution (1:5) and this amount increased by 0.2–0.4 ml. in every dilution

TABLE II

Series No. of Exp.	Date of Exp., 1930	Dilutions of Normal Medium Bacterized																																					
		1:5					1:7.5					1:10					1:12.5					1:15					1:20					1:25					1:50				
		Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males
1	8/29	...	...	160	101	0	...	...	175	108	7	...	...	187	90	11	...	...	178	92	14	...	...	156	98	18	...	...	161	94	8	...	...	165	93	7	...	...	131	100	0
2	8/29	...	...	155	94	0	...	...	183	102	8	...	...	181	95	11	...	...	156	95	5	...	...	177	102	3	...	...	178	120	14	...	...	122	106	16	...	...	137	89	11
3	8/31	...	...	176	115	0	...	...	170	104	0	...	...	172	89	11	...	...	168	102	12	...	...	159	130	0	...	...	144	99	1	...	...	175	89	11	...	...	150	86	14
4	9/11	...	...	157	100	0	...	...	172	115	0	...	...	152	100	0	...	...	183	125	0	...	...	196	89	11	...	...	144	101	0	...	...	118	99	4	...	...	141	104	5
5	11/17	10	16	121	109	0	10	18	120	111	0	10	12	119	108	0	12	9	109	107	0	8	10	103	103	0	8	10	122	104	0	9	14	110	106	0	9	10	99	93	6
6	11/17	10	10	112	104	0	10	10	103	103	0	10	10	132	124	0	8	10	110	104	0	8	14	114	106	0	12	10	126	101	2	9	12	93	93	0	5	14	72	72	0
7	11/19	10	18	123	123	0	10	18	132	132	0	10	10	136	136	0	9	20	139	139	0	9	16	124	124	0	10	16	117	117	0	10	16	133	133	0	9	14	98	98	0
8	11/19	10	16	105	105	0	10	18	131	131	0	9	12	116	116	0	10	14	133	133	0	8	18	117	115	0	10	18	169	162	0	10	16	132	132	0	9	18	114	95	16
9	12/2	5	20	49	49	0	8	18	82	82	0	6	14	59	59	0	5	14	64	64	0	8	16	80	80	0	8	18	91	91	0	9	12	80	80	0	6	14	43	43	0
10	12/2	8	20	134	106	0	10	16	138	124	0	5	16	101	101	0	...	...	138	138	0	...	...	94	94	0	...	...	20	20	0	9	10	55	55	0	9	10	129	118	0
11	12/6	10	16	121	105	0	10	18	135	109	0	10	22	164	102	0	10	10	125	109	0	10	20	119	103	0	10	22	117	100	0	10	22	111	107	0	10	20	34	34	0
12	12/6	10	22	131	110	0	10	22	126	104	0	8	20	112	100	0	9	22	124	100	0	8	22	116	100	0	9	20	105	103	0	9	22	110	107	0	7	18	80	80	0
13	12/10	10	24	105	105	0	10	22	102	102	0	10	20	102	102	0	10	20	92	92	0	9	18	90	90	0	10	20	89	89	0	10	20	97	94	3	10	18	54	51	3
14	12/10	5	12	95	95	0	10	22	106	96	10	10	18	83	83	0	10	14	76	76	0	10	22	71	69	2	10	14	95	95	0	...	...	47	35	12	10	14	56	35	21
15	12/22	7	22	104	104	0	9	26	134	113	0	10	24	133	107	0	9	24	119	101	0	10	22	141	109	0	10	22	151	124	0	10	18	115	115	0	10	16	98	98	0
Total mothers surviving				95					107					98					92					88					97					95					94		
Average second eggs per mother				17.83					18.85					16.24					15.56					18.02					16.94					16.31					15.19		
Total young				1,848					2,009					1,949					1,914					1,857					1,829					1,663					1,436		
Total females				1,525					1,636					1,512					1,577					1,512					1,520					1,444					1,196		
Total males				0					25					33					31					34					25					53					76		
Percentage of males				0					1.50					2.13					1.92					2.19					1.61					3.54					5.97		
Average young per mother				12.63					12.23					12.82					11.85					12.21					12.18					10.90					9.32		

Series No. of Exp.	Date of Exp., 1930	Dilutions of Normal Medium Bacterized											
		1:100					1:200						
		Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males		
1	8/29	...	...	166	100	10	...	...	161	127			
2	8/29	...	...	157	115	13	...	...	166	138			
3	8/31	...	...	148	101	7	...	...	173	106			
4	9/11	...	...	142	93	15	...	...	148	96			
5	11/17	7	10	83	83	0	10	10	104	104			
6	11/17	7	12	63	55	3	9	14	66	66			
7	11/19	10	12	139	123	16	7	20	56	49			
8	11/19	10	18	135	106	29	8	18	128	76			
9	12/2	8	16	62	62	0	8	10	63	63			
10	12/2	8	14	110	108	0	9	16	110	110			
11	12/6	10	16	108	102	0	8	20	69	69			
12	12/6	9	12	76	76	0	8	10	48	48			
13	12/10	10	14	71	71	0	10	14	67	67			
14	12/10	10	10	72	72	0	No Release						
15	12/22	10	12	107	97	10	8	12	83	75			
Total mothers surviving				99					85				
Average second eggs per mother				13.35					14.23				
Total young				1,639					1,442				
Total females				1,364					1,194				
Total males				103					101				
Percentage of males				7.02					7.79				
Average young per mother				10.36					9.34				

TABLE II

DILUTIONS OF NORMAL MEDIUM BACTERIZED

		1:12.5					1:15					1:20					1:25					1:50					1:100					1:200					P. W.				
	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males
9	11	...	...	178	92	14	...	...	156	98	18	...	...	161	94	8	...	...	165	93	7	...	...	131	100	0	...	...	166	100	10	...	...	161	127	15	...	...	108	93	7
3	11	...	...	156	95	5	...	...	177	102	3	...	...	178	120	14	...	...	122	106	16	...	...	137	89	11	...	...	157	115	13	...	...	166	138	5	...	...	121	96	11
9	11	...	...	168	102	12	...	...	159	130	0	...	...	144	99	1	...	...	175	89	11	...	...	150	86	14	...	...	148	101	7	...	...	173	106	6	...	...	132	109	23
8	0	...	...	183	125	0	...	...	196	89	11	...	...	144	101	0	...	...	118	99	4	...	...	141	104	5	...	...	142	93	15	...	...	148	96	16	...	...	110	110	0
8	0	12	9	109	107	0	8	10	103	103	0	8	10	122	104	0	9	14	110	106	0	9	10	99	93	6	7	10	83	83	0	10	10	104	104	0	...	...	68	59	9
4	0	8	10	110	104	0	8	14	114	106	0	12	10	126	101	2	9	12	93	93	0	5	14	72	72	0	7	12	63	55	3	9	14	66	66	0	...	...	46	40	6
6	0	9	20	139	139	0	9	16	124	124	0	10	16	117	117	0	10	16	133	133	0	9	14	98	98	0	10	12	139	123	16	7	20	56	49	7	...	...	27	27	0
6	0	10	14	133	133	0	8	18	117	115	0	10	18	169	162	0	10	16	132	132	0	9	18	114	95	16	10	18	135	106	29	8	18	128	76	44	...	...	67	67	0
9	0	5	14	64	64	0	8	16	80	80	0	8	18	91	91	0	9	12	80	80	0	6	14	43	43	0	8	16	62	62	0	8	10	63	63	0	10	12	103	95	8
1	0	...	...	138	138	0	...	...	94	94	0	...	...	20	20	0	9	10	55	55	0	9	10	129	118	0	8	14	110	108	0	9	16	110	110	0	10	12	126	120	6
2	0	10	10	125	109	0	10	20	119	103	0	10	22	117	100	0	10	22	111	107	0	10	20	34	34	0	10	16	108	102	0	8	20	69	69	0	9	8	84	84	0
0	0	9	22	124	100	0	8	22	116	100	0	9	20	105	103	0	9	22	110	107	0	7	18	80	80	0	9	12	76	76	0	8	10	48	48	0	6	10	72	72	0
2	0	10	20	92	92	0	9	18	90	90	0	10	20	89	89	0	10	20	97	94	3	10	18	54	51	3	10	14	71	71	0	10	14	67	67	0	10	14	70	70	0
3	0	10	14	76	76	0	10	22	71	69	2	10	14	95	95	0	...	...	47	35	12	10	14	56	35	21	10	10	72	72	0	No Release					10	14	98	98	0
7	0	9	24	119	101	0	10	22	141	109	0	10	22	151	124	0	10	18	115	115	0	10	16	98	98	0	10	12	107	97	10	8	12	83	75	8	10	12	27	19	8
				92					88					97					95					94					99					85					65		
				15.56					18.02					16.94					16.31					15.19					13.35					14.23					11.87		
				1,914					1,857					1,829					1,663					1,436					1,639					1,442					1,259		
				1,577					1,512					1,520					1,444					1,196					1,364					1,194					1,159		
				31					34					25					53					76					103					101					.78		
				1.92					2.19					1.61					3.54					5.97					7.02					7.79					6.30		
				11.85					12.21					12.18					10.90					9.32					10.36					9.34					8.92		

thereafter, including the pond water. It was found quite unnecessary to take the exact proportion of bacteria to the dilution of the medium into account. The bacterial suspension was added in arithmetic progression, while the dilutions of the normal medium did not ascend in this order; yet the results of the experiment do not seem to be affected by this discrepancy. Later in the work it was found more advantageous to add a small quantity of bacteria when the experiment was first set up (from 0.1 to 0.2 ml. to the first dilution, increasing the amount for each dilution by 0.2 ml.) and to bacterize a second time about 12 hours later and a third time, several hours before eggs were laid, with one-half the original amount. Successive bacterizations of this type with smaller quantities are far more satisfactory than a single bacterization with a larger quantity of the bacterial suspension. As soon as the medium was bacterized, the mothers were added, as previously described, and the series of bottles incubated at the usual temperature.

The bacterization of normal medium or normal-medium filtrates is occasionally accompanied by conditions that are detrimental to the normal development of Cladocera reared under these conditions. A few bacterized experiments had to be discarded because the added bacteria spontaneously agglutinated and settled to the bottom of the bottles in a heavy scum which enmeshed the Cladocera and apparently killed them. No attempt has been made to ascertain the cause of this phenomenon other than to rule out the possibility of drastic hydrogen-ion changes in the medium being responsible for the agglutination of the bacteria. On other occasions a lytic principle (D'Herelle, 1926) has been found in the bacterized bottles, particularly in those bottles with proportionately large amounts of pond water and large numbers of bacteria. In such bottles the medium, which was highly turbid due to the initial bacterization, became crystal clear in 24–36 hours. It would seem that this lytic principle was a bacteriophage, but there is some evidence to the contrary. According to our findings, it was species non-specific, attacking all gram-negative bacteria commonly found in manure infusions, but having little, if any, effect on gram-positive organisms from the same source. All attempts to increase the virulence of this principle or to preserve it by serial transfers have failed. This lytic principle was

consistently encountered in the summer of 1929 but has appeared only infrequently since that time. Conclusions regarding the effect of the presence of this substance in normal or bacterized medium would be quite unjustified, due to the lack of sufficient evidence; but it may safely be said that it does exert some influence on the Cladocera. The writers of this paper have hesitated about involving the bacteriological aspect of the problem too greatly, but it must be borne in mind that the bacteria in such media as have been used in these experiments for the rearing of Cladocera are the chief, if not the sole, source of food for these animals (Stuart, McPherson, and Cooper, 1931), and consequently this aspect of the question cannot be avoided.

As soon as the releases occurred in the bottles, the number of mothers surviving, the number of second eggs in the brood chamber, and the total number and sex of the young were determined, as previously described. In many cases the first type of experiment described (unbacterized) was run in conjunction with the second type (bacterized); and since young from sister-mothers were used in such cases, the unbacterized dilutions constituted a control on the bacterized experiments. All experiments in Tables I and II with the same dates were set up with young from sister-mothers and the same lot of medium. The results of these experiments are tabulated in Table II and summarized in Figure 1.

In Tables I and II the subheadings under the dilutions show, respectively, the number of surviving mothers (it is at once apparent that the 10 *Moina* young used in an experimental bottle did not always release), the average number of second eggs found in the brood chambers of the individual mothers, the total number of young released, and the numbers of females and males sexed. The total numbers for all experiments of the items listed above, together with the average number of second eggs per mother, percentage of males, and average number of young released per mother, are given for each dilution at the bottom of the table.

As will be seen in Table I, there seems to be considerable variation between the different lots of media. This is substantiated by the bacteriological analyses as previously reported. In Series 1 and 2 an appreciable percentage of males was produced in dilution 1:5 and

regularly thereafter until in Series 1 it dropped again to zero in the 1:200 dilution. On the other hand, in Series 6 no males were produced until the dilution reached 1:12.5. The fact that not a single male was produced in dilutions 1:1, 1:2, and 1:3 is quite consistent with the bacteriological findings reported by Stuart and Banta (1931). One might reasonably expect to find a lower percentage of males than 8.43 in dilution 1:5, but it will be noted that this high percentage is due to the excessively high production of males by only two series, 7 and 8. The majority of the series produced no males whatsoever in this dilution. Series 7 and 8 are not at all consistent with the thirteen other series in dilution 1:5, nor are they consistent in their sex ratio in any of the subsequent dilutions. Such erratic results are not infrequently obtained when working with these animals. Figure 1 represents in graphic form the salient features of the two experiments described. It shows the average second eggs for the mothers reared in the different dilutions of the unbacterized medium, and also for the mothers reared in the bacterized dilutions. As will be noted, the number of second eggs in the concentrated unbacterized medium is only about 9 per mother, but the number increases gradually as the dilutions increase, until the greatest number is reached in the 1:5 dilution; thereafter the number falls off gradually until no second eggs whatever are found in mothers reared in the 1:150 dilution. It is evident, however, that the number of second eggs per mother varies but little in the different bacterized dilutions, though there is a tendency for the numbers to drop as the dilutions increase. It should be observed that, even though the mothers in dilutions 1:1, 1:2, and 1:5 were transferred to the normal dilution of medium (about 1:12) some 12–36 hours before the second eggs were laid, the adverse nature of the concentrated medium was expressed by the small number of eggs laid for the second clutch. This implies that some physiological injury was received by the mothers in the concentrated medium, which is expressed by the small number of eggs in the first and second broods even in the presence of more than an adequate amount of food.

Again in Figure 1 it will be observed that the total number of young released in the various dilutions of the unbacterized medium is small in the higher concentrations, increasing, as the dilution in-

creases, to a maximum in 1:10 and then falling off again as the dilutions continue to increase. But in the bacterized dilutions the total number of young plotted against the dilutions forms more nearly a straight line with a gradual fall as the dilutions increase, though at no point in the curve is the total number decreased to half the maximum number. Curves obtained for the percentage of males in the different dilutions of normal medium unbacterized and bacterized, shown in Figure 1, exhibit the same general tendencies as the two preceding curves. The percentage of males in the unbacterized medium is lowest in the concentrated dilutions, reaches a maximum height in the 1:50 dilution, and then falls off rapidly in the higher dilutions. In the bacterized medium the percentage of males in all the dilutions is quite negligible until dilutions 1:100, 1:200, and pond water are reached, where it rises perceptibly, though always less than 8 per cent. The appearance of males in the lower dilutions is sporadic, in that only a few series show males in these dilutions; but in the higher dilutions the appearance of males is more constant. Despite the presence of an adequate number of bacteria in the higher dilutions, the drop in the number of second eggs, total number of young, and the increase in male percentage might be ascribed to the insufficiency of the "food accessory" postulated by Stuart and Banta (1929). Unfortunately, there is at present no direct evidence to support this assumption, since this phase of the work has had to be abandoned for the present. Series 1, 2, 3, and 4 are experiments in which only one addition of bacteria was made. This was done at the time the experiment was set up. The ineffectiveness of this method, as compared with successive bacterizations, is easily seen (Table I).

DISCUSSION

In a series of fifteen experiments wherein *Moina* mothers were crowded in 60 ml. of various dilutions of normal medium a sex curve was obtained similar to that obtained by rearing crowded mothers in dilutions of bacterized filtrates of normal medium. In high concentrations of the normal medium only female young were produced by the crowded mothers. As the dilutions increased, the percentage of males increased, to a maximum in dilution 1:50. Further dilution, however, resulted in the reappearance of females. The percentage

of males plotted against the dilutions gives a typical curve. This also holds true for second eggs, total number of young produced, and the number of mothers releasing. In another series of experiments the same degree of crowding was used in approximately the same dilutions as in the first experiment except that the dilutions were bacterized. In these experiments the percentage of male young was negligible until in the highest dilutions, where it increased to over 7 per cent. The number of second eggs produced by these mothers was fairly constant in all but the highest dilutions, where the number dropped somewhat. This is also true for the total number of young and the number of mothers releasing. Bacterizing the dilutions seems to have restored the food value which was lost in diluting the concentrated medium with pond water. This is not strictly true in the dilutions 1:100, 1:200, and pond water, since the number of young, second eggs, and surviving mothers falls appreciably in these bottles. It would seem that, as the number of bacteria in these bottles was adequate, a possible "food accessory" in the concentrated medium was diluted beyond its effective limits. It is hoped that this "food accessory" substance can be more fully investigated in the future.

The character of the curves obtained for second eggs, total young, and percentage of males in increasing unbacterized dilutions has already been mentioned; and to these curves might be added a similar one for the percentage of mothers releasing, the number being small in the low dilutions, increasing to a maximum in dilution 1:10, then dropping again as the dilutions continue to increase. Retardation in the time required by the mothers to release in the various bottles plotted against the dilutions would give a similar curve, considerable retardation occurring in dilutions 1:1 through 1:3, mothers in dilutions 1:7.5 or 1:10 releasing first with the retardation time gradually increasing thereafter to as much as 12 hours in dilution 1:200. Again the size of the young released, together with other characteristics of the young, when plotted would present about the same type of curve. Therefore, since the number of second eggs, total number of young, number of mothers releasing, retardation in time of release, and other characteristics all follow the same general trend when plotted against the dilution, and are for the most part de-

pendent on the concentration of the medium, is it not more logical to conclude that the percentage of males, which shows a similar trend, is due to the same factor, concentration of medium, rather than to the accumulation of excretory products only from the crowded mothers, especially since the mothers are crowded to the same degree in all the dilutions?

It might be contended that the smaller number of mothers releasing in the more concentrated dilutions would be responsible for the absence or the small number of males produced in these dilutions. This would be true had the mothers failing to release died before acting as effective crowders; but the majority of mothers failing to release did not die: they merely absorbed eggs already laid. Attention should be called to the fact that in the range of dilutions (1:10–1:50) where the number of releasing mothers is consistently high, the percentage of male young is steadily increasing. In the 1:100–1:200 dilutions where the percentage of males is rapidly falling off, the number of releasing mothers likewise falls; but mothers seldom died in these dilutions: they more often fail to lay eggs or, having laid eggs, absorb them, and therefore, must have acted as effective crowders in these dilutions.

SUMMARY

1. *Moina* mothers crowded in various dilutions of normal medium produced young with approximately the same sex ratio as that of the young produced by mothers crowded in various dilutions of bacterized filtrates of normal medium.

2. Mothers crowded in bacterized dilutions of normal medium produced, for the most part, only female young.

3. Under the experimental conditions designated, food, rather than excretory substances, is favored as the effective factor in sex control.

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AVAILABLE FOOD AND CROWDING AS FACTORS INFLUENCING THE SEX OF MOINA MACROCOPA. II

(One figure)

C. A. STUART, H. J. COOPER, AND JUANITA TALLMAN
Brown University

THE problem of food as a factor in the control of sex in *Moina macrocopa* has been approached in several different ways by Stuart and Banta (1931) and Stuart, Tallman, and Cooper (1931). The former showed that when Cladocera were reared in varying dilutions of bacterized filtrates of normal medium (manure infusions) the number of males produced by crowded mothers very closely followed the number of viable bacteria present in the medium. They also found that *Moina* mothers, even in a crowded condition (3 mothers in 10 ml.) would produce only female young in the presence of an adequate number of viable, utilizable bacteria.

The latter investigators confirmed and amplified these findings by rearing crowded *Moina* mothers in dilutions of normal medium, unbacterized and bacterized. They found that in sufficiently concentrated manure infusions crowded mothers (10 in 60 ml.) produced only female young, while in more dilute concentrations males were produced in numbers roughly proportional to the dilution of the medium. In excessive dilutions, however, a reduced percentage of males or, in some experiments, no males at all were found. In the unbacterized dilutions the number of second eggs and the total number of young produced by the mothers rapidly decreased in the higher dilutions. Comparable experiments wherein *Moina* mothers were crowded to the same degree in bacterized dilutions of the same medium produced, for the most part, only female young; and the decrease in the number of second eggs and the total number of young, while apparent, was by no means as great as in the unbacterized dilutions. Obviously then, bacterizing the medium has overcome the food-dilution factor, and likewise any effect on sex con-

trol that might be obtained from crowding 10 mothers in 60 ml. of medium in these experiments.

The following experiment was devised to determine the effectiveness of bacterization in suppressing any effect that excessive crowding of mothers might exert on the sex of the young. Twelve, 18, and 24 *Moina* mothers were crowded in 75 ml. of normal medium. Furthermore, 12, 18, and 24 mothers were crowded in the same amount of the same medium to which bacteria had been added. All experimental and control bottles were incubated at 24–26° C. The bacterization can best be accomplished by the addition of approximately (depending on the general appearance of the specific batch of medium) 1 ml. of a bacterial suspension to the 12-mother bottle, 1.5 ml. to the 18-mother bottle, and 2 ml. to the 24-mother bottle. The usual suspension of *Aërobacter aërogenes* was used in these experiments (Stuart, Tallman, and Cooper, 1931). These bottles were bacterized at least twice before the first eggs were laid. Later additions were usually made in 0.5, 1, and 1.5 ml. amounts. Following each bacterization, the medium in the bottles was stirred very gently with a pipette to insure the best possible degree of dispersion of the bacteria. The control bottles (mothers crowded in the unbacterized medium) were stirred in the same manner at the same time. Control bottles were bacterized after the mothers had deposited eggs in the brood chamber; and since this in no way affects the sex and number of young, it was hoped that this procedure would facilitate counting and sexing of the young by increasing their size. No amount of bacterization after the first eggs are laid, however, will produce young from control bottles as large as those produced by mothers reared in medium bacterized from the start of the experiment. When releases occurred, the mothers were counted, and the number of second eggs, total number of young, and the sex of about 50 per cent of the young from each bottle were determined. Fifty per cent of the young were not sexed in all cases of excessive crowding, since in bottles containing several hundred young this was considered unnecessary. In all experiments, however, more than 100 young were sexed from each bottle.

It was found after several such experiments that the sex of the young could be quite easily controlled by bacterization even in the

more heavily crowded bottles, since no males were produced in the majority of the bottles of the bacterized medium. The crowding was then increased—30, 36, 42, and 48 mothers being crowded in the same amount of medium (75 ml.) of both unbacterized and bacterized medium.

This set of experiments was originally designed to include fifteen series with 12 mothers, ten series with 18, 24, and 30 mothers, and five with 36, 42, and 48 mothers per bottle; and it was not noted until a general tabulation of these experiments had been made that the male percentage in the control (unbacterized) bottles was not at all similar to what had been obtained by other investigators working along similar lines. Banta and Brown (1929*a*, 1929*b*) reported higher male percentages from 12 to 36 mothers crowded in 75 ml. of medium than our experiments show, and they concluded that the percentage of males was proportional to the crowding and to the retardation of the mothers.[1] A similar result was anticipated in this work since in previous experiments it had been found that crowding 3, 6, 9, and 12 mothers in 60 ml. of medium gave a percentage of males more or less proportional to the degree of crowding. But, as will be seen in Table I and Figure 1, the percentage of males obtained from these experiments remained fairly constant in crowding from 12 to 48 mothers in 75 ml. of medium. It was therefore considered advisable to continue crowding mothers in normal medium (unbacterized) to obtain additional data. These additional experiments will be found proportionately designated in Table I.

The male percentage was not altered to any appreciable extent by the additional experiments. In view of this, it would seem that the male percentage obtained from crowded *Moina* mothers is proportional to the crowding, but only to a certain degree of crowding. Considering for the moment only the control experiments (unbacterized), we find certain evidence in these experiments which tends to support the observation that while in moderate crowding the male percentage may be proportional to the number of mothers per bottle,

[1] Since the writing of this paper we have been informed in a personal communication from Banta and Brown that the probable explanation of the discrepancy between their work and ours lies in the fact that their experiments in crowding were carried out at 17° C.

in excessive crowding this need not necessarily hold true. As has already been pointed out, when in any experiment involving the diminution of food the male percentage reaches from 25 to 30 per cent,

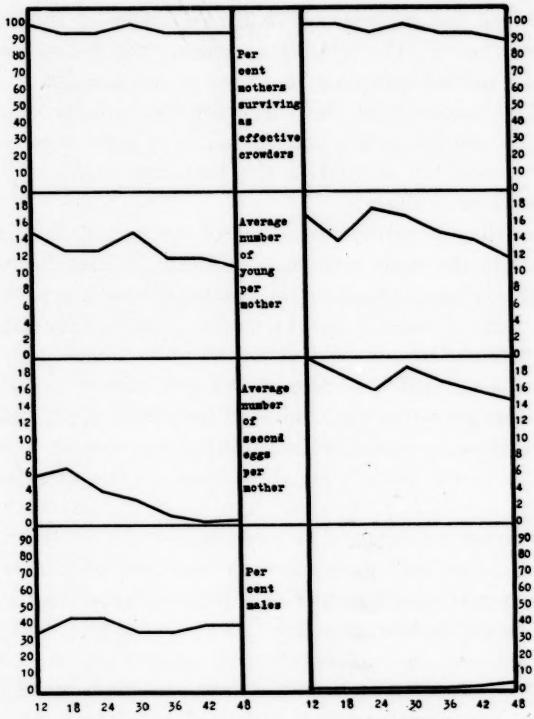
Per cent mothers surviving as effective crowders
Average number of young per mother
Average number of second eggs per mother
Per cent males
Unbacterized Medium
Bacterized Medium
Number of Mothers in 75 Ml. of Medium

FIG. 1.—Graphic presentation of the results of the experiments tabulated in Table I. The various curves show the relationship between the mothers surviving as effective crowders, average young and second eggs per mother, and the percentage of males obtained from 12 to 48 mothers (ordinates) reared in 75 ml. of unbacterized medium (left) and from 12 to 48 mothers in 75 ml. of bacterized medium (right).

further increase in male percentage is accompanied by a corresponding decrease in the number of young produced in such an experiment. The male percentage obtained from crowding 12–48 mothers

in 75 ml. of medium remaining fairly constant should then be accompanied by a similar constancy in the number of young produced. This constancy is shown in Table I and Figure 1. The number of mothers surviving as effective crowders also varies but little, in that practically all the mothers survive irrespective of the number of mothers per bottle. The steadily decreasing number of second eggs per mother as the crowding increases is not consistent with the straight-line tendency of the curve for the number of surviving mothers, number of young per mother, and male percentage, but does not necessarily invalidate the tentative explanation that is offered for these findings.

The fact that 48 mothers in 75 ml. of medium do fully as well as 12 mothers in the same amount of medium, in that the number of mothers surviving as crowders, the number of young per mother, and the percentage of males is about the same, immediately suggests the possibility that some diverse effects of animal aggregation (Allee, 1931) are being exhibited here. A few preliminary experiments in which further crowding was employed (namely, 72, 96, and 150 animals in 75 ml. of medium) produced sufficient evidence to warrant a continuance of the work along these lines. In one experiment (unbacterized) in which 72 mothers were used, 72 surviving mothers produced no second eggs and an average of 12.65 young per mother, of which 41.29 per cent were males; in bacterized medium 72 surviving mothers produced 8 second eggs per mother and an average of 12.16 young per mother, of which 14.12 per cent were males. In another experiment (unbacterized) with 96 mothers a total of 735 young were released, of which 34.41 per cent were males; while 96 mothers in bacterized medium produced a total of 186 young, of which 11 per cent were males (number of surviving mothers not noted). Other experiments of this type, however, demonstrated that a procedure quite different from that described in this paper must be employed before mothers crowded to this extent could be reared effectively. Further consideration of this question is reserved.

It is questionable whether the increase in the percentage of males in the 12-mother bottle from 35.97 to 43.94 per cent in the 18-mother bottle is any more significant than the subsequent decrease in the 24-mother bottle from 44.72 per cent to 32.48 per cent in the 30-

TABLE I

| Series No. of Exp. | Date of Exp., 1930 | Number of Mothers in 75 Ml. of Normal Medium | | |

TABLE I

NUMBER OF MOTHERS IN 75 ML. OF NORMAL MEDIUM

Males	24										30										36						
	Control						Bacterized				Control						Bacterized				Control						
	Date Add. Exp., 1931	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Date Add. Exp., 1931	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Date Add. Exp., 1931	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving
0	....	23	1	268	88	68	24	18	237	132	0	1/2	30	2	342	108	104	...	...	...	...	...	1/2	36	2	394	17	144	...
11	....	24	4	301	88	94	24	14	391	189	27	1/5	30	4	335	189	20	...	...	...	...	...	1/5	33	2	421	174	52	...
14	....	24	12	228	44	156	21	14	351	246	3	1/5	27	2	301	173	23	...	...	...	...	...	1/5	34	0	397	175	13	...
0	....	24	12	257	38	80	24	12	392	223	0	1/9	31	4	393	114	94	...	...	...	...	...	1/9	34	0	379	39	137	...
0	....	16	0	274	96	60	23	14	444	212	0	1/9	30	6	359	101	92	...	...	...	...	...	1/9	36	2	408	98	59	...
4	....	23	2	196	131	56	24	20	466	365	0	1/11	27	4	501	295	79	...	...	...	...	...	1/10	36	2	451	115	48	...
2	....	24	2	313	99	48	22	18	481	230	0	1/11	29	1	509	159	200	...	...	...	...	...	1/10	33	0	462	116	34	...
0	....	24	6	111	93	4	24	16	414	108	0	1/11	30	2	441	138	35	...	...	...	...	...	1/10	35	1	475	146	63	...
0	....	24	2	329	133	34	23	18	537	376	0	....	29	1	483	136	92	28	16	555	336	13	1/12	...	...	416	145	41	...
0	....	24	2	347	46	114	24	20	439	196	9	....	29	1	539	140	66	27	16	544	387	0	1/12	...	...	499	125	38	...
...	1/2	24	6	404	77	166	...	...	...	...	...	....	30	4	228	209	19	30	16	158	106	0	....	36	2	547	205	35	34
...	1/5	24	4	324	172	0	...	...	...	...	...	....	30	0	453	146	159	30	18	637	437	4	....	34	1	462	169	51	35
...	1/5	24	4	349	183	0	...	...	...	...	...	....	27	2	389	71	54	30	18	402	290	0	....	31	2	297	168	72	33
...	1/9	22	6	357	41	122	...	...	...	...	...	....	30	8	582	176	129	30	22	613	365	22	....	33	0	407	133	27	30
...	1/9	23	2	384	65	126	...	...	...	...	...	....	30	4	559	227	33	30	24	576	139	0	....	36	1	453	14	192	30
			347					233						439					205					447				
			4.44					16.42						3.02					18.63					1.16				
			4,442					4,152						6,414					3,485					6,468				
			1,394					2,277						2,382					2,060					1,839				
			1,128					39						1,199					39					1,006				
			44.72					1.68						33.48					1.85					35.36				
			12.80					17.81						14.61					17.00					12.42				

6							42															48													
	Bacterized						Control						Bacterized						Control						Bacterized					
Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Date Add. Exp., 1931	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Date Add. Exp., 1931	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males	Mothers Surviving	Av. Second Eggs per Mother	Total Young	Females	Males
44	...	...	...	...	...	1/2	41	0	485	58	189	...	...	...	...	...	1/2	46	0	547	141	104	...	...	...	...	...
52	...	...	...	...	...	1/2	40	2	502	60	127	...	...	...	...	...	1/2	48	0	509	80	176	...	...	...	...	...
13	...	...	...	...	...	1/5	40	0	541	194	128	...	...	...	...	...	1/5	48	0	465	22	185	...	...	...	...	...
37	...	...	...	...	...	1/5	42	0	467	36	219	...	...	...	...	...	1/5	45	0	480	92	121	...	...	...	...	...
59	...	...	...	...	...	1/9	39	2	444	148	5	...	...	...	...	...	1/9	44	0	420	158	6	...	...	...	...	...
48	...	...	...	...	...	1/9	38	2	485	132	1	...	...	...	...	...	1/9	45	0	398	186	13	...	...	...	...	...
34	...	...	...	...	...	1/10	42	0	484	119	138	...	...	...	...	...	1/10	47	0	518	177	66	...	...	...	...	...
63	...	...	...	...	...	1/10	40	0	453	186	139	...	...	...	...	...	1/10	46	0	509	138	53	...	...	...	...	...
41	...	...	...	...	...	1/12	...	...	494	201	47	...	...	...	...	...	1/12	...	...	430	180	52	...	...	...	...	...
38	...	...	...	...	...	1/12	...	...	541	261	32	...	...	...	...	...	1/12	...	...	577	208	68	...	...	...	...	...
35	34	18	531	240	0	....	42	0	521	176	21	41	18	533	260	0	....	48	0	457	125	18	42	16	629	356	0
51	35	16	492	219	4	....	41	0	477	126	20	40	16	580	301	2	....	47	0	495	174	79	49	14	574	251	25
72	33	16	414	213	0	....	42	0	475	146	16	40	18	587	209	7	....	48	6	577	188	74	44	16	628	399	28
27	36	16	571	171	3	....	41	0	464	123	17	41	16	616	235	19	....	48	0	665	87	99	46	16	443	136	1
92	36	18	551	282	15	....	42	0	503	113	223	41	14	602	254	20	....	47	2	490	17	98	38	14	410	134	22
			174						530					203						607					219		
			16.80						.44					16.39						.62					15.20		
			2,559						7,336					2,918						7,537					2,684		
			1,125						2,079					1,259						1,973					1,276		
			22						1,332					48						1,212					76		
			1.91						39.05					3.67						38.05					5.62		
			14.70						11.88					14.37						10.75					12.25		

mother bottle. It should be borne in mind that considerable variation in the number of males obtained from mothers crowded to the same degree occurs even in the same medium. In Table I it will be noted that the 36-mother bottle in Series 14 produced but 16.87 per cent males, while in Series 15 the 36-mother bottle produced 93.20 per cent males; yet the mothers crowded in both bottles were from sister-mothers and medium from the same lot was used in both series.

The results obtained from crowding 12–48 mothers in 75 ml. of bacterized medium needs little discussion. Male production is entirely suppressed in many of the experiments, and considerably reduced in comparison to the controls in all others. Another notable difference between the bacterized and unbacterized experiments is found in a comparison of the curves obtained for second eggs. It would seem from the sharp decline of second eggs in the unbacterized medium that, while excessively crowded mothers can maintain what appears to be a normal existence sufficiently long to lay first eggs, it does not continue indefinitely. An interesting and profitable field of investigation is to be found in a study of the young developing from these second eggs in both environments (bacterized and unbacterized media).

DISCUSSION

In a series of experiments in which 12–48 mothers were crowded in 75 ml. of medium, a male percentage increasing with the crowding, together with a decrease in the average number of young per mother, was anticipated. It was found, however, that the percentage of males and the average number of young per mother remained practically constant despite the number of mothers per bottle. Additional experiments using 72 and 96 mothers in 75 ml. of medium tend to confirm these findings. The tentative explanation is advanced that other effects of animal aggregation are being experienced in these bottles. Further discussion is reserved for a subsequent paper.

From the evidence produced in this and two preceding papers it would seem that, under the conditions designated, the sex of the young produced by crowded *Moina* mothers could be controlled by varying the number of viable and utilizable bacteria in the medium in which the mothers are reared. Furthermore, mothers crowded to

the extent of 12–48, and even 96 in 75 ml. of medium, produced for the most part only female young when the bacterial content of the medium was sufficiently increased with a suitable species of bacterium.

Strongly as this evidence may suggest that the bacteria as available food have been the determining factor in the control of sex in these experiments, we are not yet prepared to make this a definite assertion. There are other possible explanations for the rôle of bacteria here; for example, if Cladocera produce excretory substances which do control or influence sex, it is possible that the physiological activities of the bacteria might in some way break down these substances. Even this explanation loses much of its force, since it has been reported that the excretory substances of a great variety of aquatic animals will also influence the sex of young produced by Cladocera (Banta and Brown, 1929*a*), and it is not unreasonable to suppose that the excretory substances of bacteria might also act the same way. The assertion that excretory substances do influence sex in *Moina* is based on indirect evidence and is open to the same criticism that might be made against any assertive statement regarding the rôle of bacteria in the control of sex in *Moina*. In a subsequent paper it is planned to deal more explicitly with both aspects of this problem.

In our opinion, the whole question of sex control in these animals cannot at all times be limited to any such simple explanation as the mere presence or absence of excretory substances, or to the mere presence or absence of sufficient bacteria as available food. Under certain experimental conditions, either one of these factors may be invoked to explain the control of sex, that is, they may function as an immediate cause acting on the mother directly; but under certain other conditions, they can at best function only as a remote cause acting indirectly, or acting in conjunction with some other factor, as, for example, temperature. It is not infrequently noted that a single mother in both an adequate amount of medium (75–100 ml.) and in the presence of an adequate amount of food will produce male young; while, on the other hand, crowded mothers in an inadequate amount of medium and food may produce only females. It is admitted that a single mother in the presence of sufficient medium and food, but

reared at low temperature, may produce males; and there is some evidence to show that this also occurs at unusually high temperatures. Many other experiments likewise point to the fact that any retardation in the development (lowering the rate of metabolism) is associated with male production, and at times in this work it has been felt that the production of males by a single mother in a favorable environment might be an expression of some physiological injury received by the mother in a previous environment. This injury may retard the development (metabolism) of the mother even under most favorable conditions, and results in the production of male young. There is little, if any, evidence at the present time to support this contention, but the assumption seems all the more tenable in view of certain difficulties that have been encountered in maintaining stock cultures of Cladocera.

Not infrequently have our stock cultures of these animals seemed to experience sporadic periods of decline. In such a period the mothers are sluggish, releasing extremely small-sized young, with occasionally as few as 2 or 3 young to the clutch; and many of the young and mothers die. It has been noted that these periods of decline are frequently introduced (possibly induced) by unusually large releases, each mother releasing as many as 48–52 young, of which many are males. On some occasions periods of decline are unquestionably brought about by unfavorable factors in the medium, such as the presence of deleterious bacteria. But at other times analyses of the medium show a bacterial flora which, both in quality and quantity, seems to be quite normal. It has also been noted that the young from sister-mothers reared in the same medium under identical conditions do not always react the same, the young from one mother going into a period of decline while the young from another mother in the same batch of medium maintain their usual vigor. For a period of over two months in the summers of 1929 and 1930 all work was interrupted because every effort to maintain a normal stock failed. Samples of pond water from several different sources, different lots of garden soil and manure, fresh and dried for varying times, were tried, but without success. It has been our experience that when the Cladocera are going through a period of this type there is little that can be done to restore them to their normal

vigor. The animals appear to return to their normal condition without any perceptible change having taken place in their environment. As a matter of fact, a certain line will be found restored while another is still in the decline phase in the same lot of medium. During such periods of depression it has been found beneficial to carry some of the stock in very dilute medium, since the mortality is low in such an environment. The young produced under such conditions are not, however, satisfactory for experimental work of the type reported in this paper.

In all of this work it has been found that while the animals are in a period of depression all experimental results are apt to be quite different from those obtained under more favorable conditions. There seems to be a decided tendency on the part of *Moina* mothers during this period to produce female young irrespective of the conditions under which they are reared. On two occasions it was found that mothers in the decline period when crowded 30–48 in 75 ml. of medium produced only female young in every bottle. We frankly admit that such experiments have been discarded and not included in the table. This, at first thought, might not seem justified; but since it is granted that there are occasions when the sex of *Moina* young cannot be controlled by the specified environmental conditions, it seems justifiable to limit our experimental data to the results obtained with normal mothers. But until such time as more is known about the relation of these animals to their environment, such peculiarities serve to emphasize the danger of drawing too inclusive conclusions.

Why an apparently normal mother reared in the presence of an adequate amount of medium, an adequate number of bacteria, and at the usual temperature should produce male young cannot be explained on the basis of any work yet done on these animals. We are aware that internal rhythms have been invoked to explain such sex cycles. This theory has seemed attractive when, on several occasions, periods of decline have been foreshadowed by the appearance of males in stock cultures. Yet we feel that always some external agent altering the physiological activities of the mothers, which in turn regulate sex, is the more probable explanation. It is possible that at some later date this subject will be reported in more detail.

We wish to acknowledge Dr. W. A. Castle's valuable assistance in preparing the manuscript for this series of papers.

SUMMARY

1. Twelve to 96 mothers crowded in 75 ml. of medium produced about the same percentage of males; about the same percentage of mothers survive as effective crowders; and the average number of young per mother is approximately the same irrespective of the degree of crowding.

2. Evidence in support of other physiological effects of animal aggregation is pointed out.

3. Twelve to 96 mothers crowded in 75 ml. of bacterized normal medium for the most part produce female young.

4. It is concluded that conditions exist under which neither excretory substances nor the quantity of available food can be ascribed as the immediate factor in the control of sex in *Moina*.

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RESPIRATORY METABOLISM DURING PUPAL DEVELOPMENT OF GALLERIA MELLONELLA (BEE MOTH)

(Ten figures)

IVON R. TAYLOR AND H. B. STEINBACH
Biological Laboratory, Brown University

INTRODUCTION

DURING the development of a pupa, striking structural and physiological changes occur within the insect. Several investigators have shown that in many different species of insects the rates of oxygen consumption and carbon-dioxide output change in the pupal stage as metamorphosis progresses. The rates of oxygen consumption of pupae of a few species have been reported graphically by one of the present writers (Taylor, 1927), whose results indicated the possibility of the existence of a sex difference in the rates of oxygen consumption of the lepidopterous pupae used, i.e., *Ephestia kuehniella* and *Galleria mellonella*. The work here reported was undertaken to determine definitely whether or not such a sex difference actually exists in *G. mellonella* and for the purpose of determining the carbon-dioxide production and the respiratory quotients throughout the pupal period, in this species.

Among the investigators who have measured the rates of oxygen consumption or of carbon-dioxide production or of both of pupae throughout pupal development are Krogh (1914), Tangl (1909), Weinland (1905), Bodine and Orr (1925), Fink (1925), Clare (1925), Taylor (1927), and Frew (1929). All these workers found that the rates of oxygen consumption or of carbon-dioxide output of the pupae studied are high at first, decrease to a minimum, and then rise to a maximum value. For the lepidopterous pupae *Ephestia kuehniella* and *Galleria mellonella* the oxygen curve was found by Taylor (1927) to be of this general U-shaped type, but always modified by the presence of an additional decline in the curve just before emergence of the pupa.

MATERIAL AND METHODS

The pupae of *Galleria mellonella* were used exclusively throughout the following experiments and were taken from cultures kept in a dark incubator at 30° C. in the laboratory. The larvae of these bee moths were fed on old honeycombs from which most of the honey had been extracted. The major part of the diet of this animal is probably the wax of the honeycombs. When pupae were required for investigation, a few larvae about ready to pupate were placed in small jars with an abundant food supply and were examined frequently so that in the case of each individual the time at which pupation occurred could be determined to within 3 or 4 hours at the most. In this way it was possible, and was the practice, to commence measurements of the gaseous exchange from 1 to 4 hours after the act of pupation.

A short time before pupation, the bee-moth larva stops feeding and spins a white cocoon about itself. After pupation the pupa case quickly forms; and the pupa, at first a light cream color, soon becomes reddish and then dark brown. The pupa case is segmented and somewhat rigid. The pupal period during which, of course, the exchange of material between animal and the environment involves only gases is of about $7\frac{1}{2}$ days duration at 30° C. Throughout this period the pupa is in a quiescent condition and remains motionless if undisturbed. The measurements of the gas exchange, therefore, may be regarded as taken under conditions of basal metabolism.

In these experiments the oxygen consumption and carbon-dioxide production were measured by means of sensitive differential manometers of the general type used and described by Krogh (1915) but having the improvements introduced by Bodine and Orr (1925). Kerosene was used as the manometer fluid. All measurements were made upon individual pupae and not upon pupae taken as a group. This was possible on account of the sensitivity of the manometers and the fact that a pupa is fairly large (about 0.1 gram in weight). In this way errors due to differences in the rate of development of different individuals were avoided. The curves which were obtained representing oxygen consumption and carbon-dioxide production are those for individual pupae. The manometers were calibrated so that the rates of oxygen consumption and carbon-dioxide output could

be expressed in terms of cubic millimeters per gram of pupa per hour.

Each pupa, before being placed in its manometer, was removed from its white cocoon, which fits very loosely over the animal. The pupa was then weighed and placed head downward in a small cotton gauze basket suspended from a small glass hook built into the shank of the manometer supporting the manometer bulb. While measurements were in progress, the manometer bulbs were kept immersed in the water of a large water bath automatically controlled so that the temperature was kept constant at 30° C. to within a small fraction of a degree. This temperature was used in all the experiments described in this paper. In the manometer bulbs the pupae completed their development successfully and emerged as normal moths.

The pupa case becomes gradually chitinized during pupal development, and so probably cannot be regarded as being entirely composed of actively respiring tissue throughout the entire period of metamorphosis. However, in calculating the rates of oxygen consumption and carbon-dioxide production the weight of the pupa case was in every instance included in the weight of the animal. This was done because it is not possible to determine at any given time the exact extent to which the pupa case may be regarded as actively respiring tissue. Any error caused by this method of treatment would be slight on account of the fact that the pupa case weighs only about 1 mg., whereas the average weight of the pupa is about 0.1 gm.

Each pupa was placed in its manometer as soon as possible after the act of pupation, and the measurements of oxygen consumption or of carbon-dioxide output were made practically continuously day and night throughout the metamorphosis of each pupa. These measurements were only interrupted by short periods at regular intervals during which the animals were weighed, fresh air admitted to the manometer bulbs, and the KOH solution introduced into or removed from the bulbs. The duration of each of these short intervals during which the readings were interrupted was usually half an hour.

THE OXYGEN CONSUMPTION

Measurements of oxygen consumption were made on 29 female and 19 male pupae individually. Each animal was placed in its

manometer from 1 to 4 hours after the commencement of the pupal period, and the measurements were made continuously until emergence except for the short periodic interruptions mentioned above.

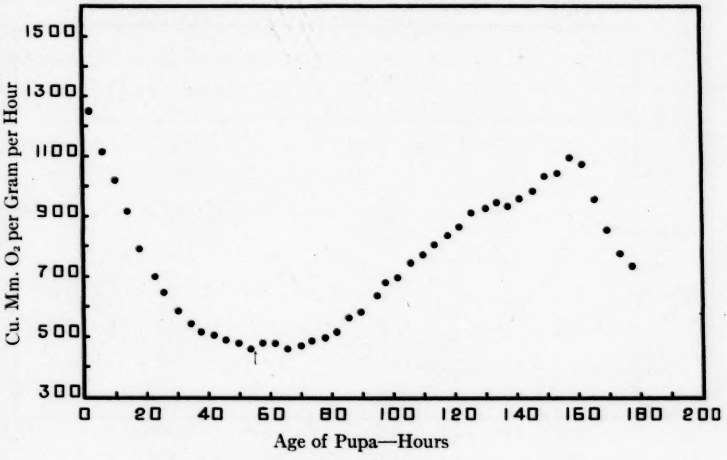

FIG. 1.—Oxygen consumption of male bee-moth pupa. Temperature, 30° C.

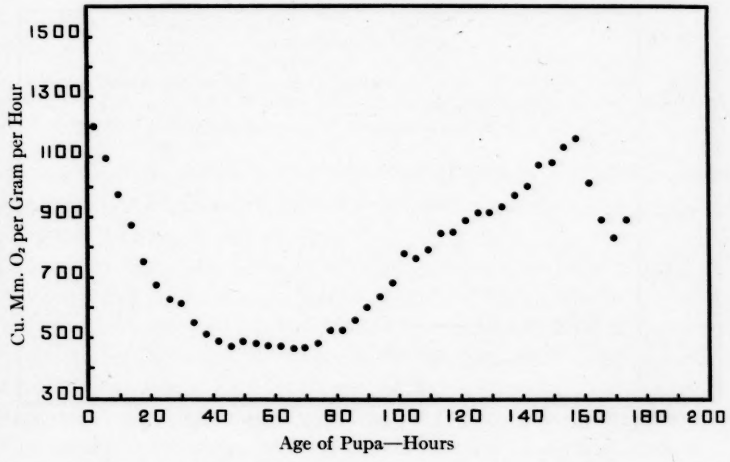

FIG. 2.—Oxygen consumption of male bee-moth pupa. Temperature 30° C.

Each time interval during which measurements were made between interruptions was of 3 hours and 30 minutes' duration. In this series of experiments manometer bulbs each having a capacity close to 55 cc. were used.

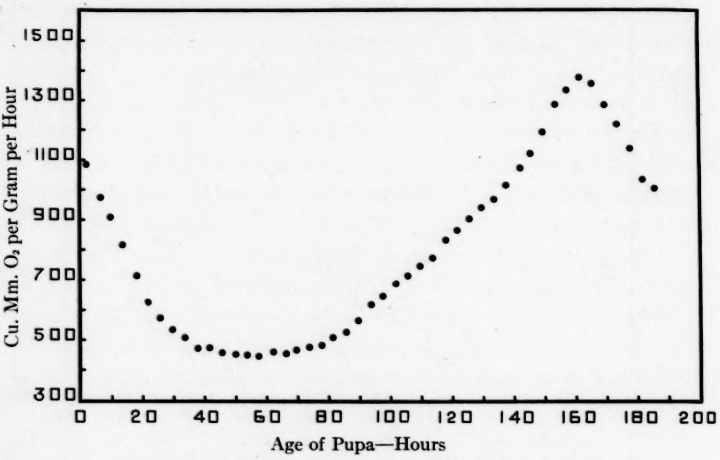

FIG. 3.—Oxygen consumption of female bee-moth pupa. Temperature, 30° C.

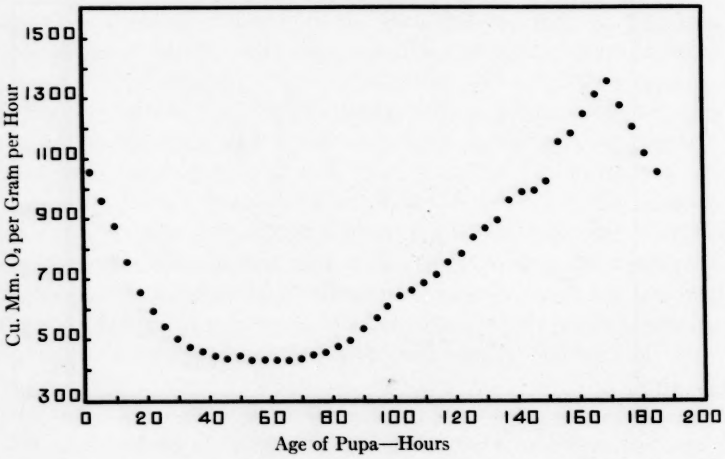

FIG. 4.—Oxygen consumption of female bee-moth pupa. Temperature, 30° C.

In Figures 1–4 are shown graphs indicating the rates of oxygen consumption of several pupae selected as typical. The curves for the other individuals used show practically the same regularity. The rates are expressed as the number of cubic millimeters of oxygen consumed per gram of pupa per hour. Each point on the curve represents the average rate during a $3\frac{1}{2}$-hour period. The rates up to the beginning of emergence are shown. In Figures 1 and 2 the oxygen

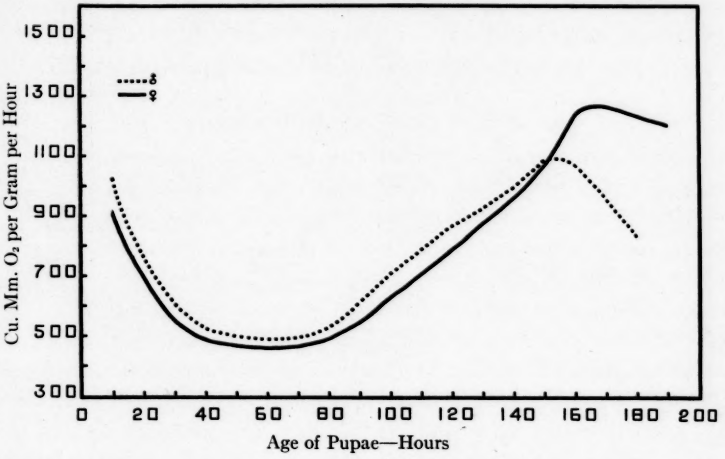

FIG. 5.—The dotted curve is the composite curve for the 19 male pupae, and the solid curve is the composite curve for the 29 female pupae. Each curve represents oxygen consumption of bee-moth pupae at a temperature of 30° C.

curves for two different individual male pupae are represented. Corresponding curves for two different female bee moth pupae are shown in Figures 3 and 4.

It is evident from these curves that those of the females tend to reach a lower minimum value than the curves for the males and that the curves for the females reach a higher maximum point than those of the males. This sex difference is brought out more clearly in Figure 5, which shows the composite oxygen curve for the 29 female pupae and one for the 19 male purpae used in this part of the work. The points on the curves in Figure 5 represent arithmetical averages.

In Table I are indicated the values of the minimum rate and the

maximum rate of oxygen consumption of each pupa used. The figures represent the number of cubic millimeters of oxygen consumed per gram of pupa per hour.

TABLE I

The figures represent cubic millimeters of oxygen consumed per gram of pupa per hour.

Pupa No.	Minimum Oxygen	Maximum Oxygen	Pupa No.	Minimum Oxygen	Maximum Oxygen
		Male Pupae			
1	521	1,155	13	465	1,070
2	517	1,191	14	451	1,154
3	540	1,185	15	503	1,250
4	542	1,311	16	469	1,132
5	453	1,247	17	465	1,163
6	453	1,218	18	454	1,073
7	464	1,163	19	470	1,226
8	516	1,228			
9	450	1,106	Average	480.8	1,176.9
10	476	1,259	Lowest	450	1,070
11	461	1,096	Highest	542	1,311
12	466	1,135			
		Female Pupae			
20	474	1,462	37	418	1,224
21	437	1,428	38	447	1,340
22	473	1,451	39	460	1,345
23	441	1,525	40	425	1,296
24	453	1,382	41	481	1,332
25	524	1,550	42	465	1,478
26	446	1,523	43	491	1,472
27	458	1,494	44	460	1,336
28	457	1,413	45	391	1,296
29	424	1,373	46	445	1,343
30	447	1,494	47	457	1,310
31	434	1,350	48	455	1,520
32	436	1,343			
33	507	1,487	Average	452.7	1,406.3
34	446	1,379	Lowest	391	1,224
35	432	1,360	Highest	524	1,550
36	444	1,478			

These figures clearly indicate that the average minimum rate of oxygen consumption for the males is greater than that for the female pupae and that the average maximum rate for the females is greater than that for the males. By comparing the maximum rates of oxygen consumption of the females with those of the males, it

has been found that the difference between their means is 3.19 times its probable error, and accordingly this sex difference can be regarded as statistically significant. The difference in the minimum rates of males and females does not appear to be statistically significant. However, the fact that the composite oxygen curve shown in Figure 5, the one shown in Figure 10 later in this paper for other pupae, and the composite carbon-dioxide curve in Figure 10 all show higher minimum rates for the males than for the females, indicates the considerable probability that there also exists a biologically significant sex difference with respect to the minimum rates of oxygen consumption.

CARBON-DIOXIDE PRODUCTION

Measurements of the rates of carbon-dioxide production were also made by means of the differential manometer method. In this part of the work 19 pupae were used—9 males and 10 females. The measurements were made upon individual pupae; and in order to secure readings of a desirable magnitude, manometer bulbs each having a capacity close to 36 cc. were used on every manometer, and each uninterrupted period during which the manometers were set for carbon-dioxide determinations (really O_2 minus CO_2) was increased to 6 hours' duration. After each of these runs, there was an interval of half an hour during which the bulbs were removed from the manometers so that the air could be renewed, KOH added, pupae weighed, etc. Each such interval was then followed by a $3\frac{1}{2}$-hour period for measurements of oxygen consumption, to be followed by a $\frac{1}{2}$-hour interval for renewing air in the bulbs, etc. In this way periods for measurements of oxygen consumption alternated with periods for measurement of carbon-dioxide production; each period was followed by a $\frac{1}{2}$-hour interval for renewing or changing the contents of the manometer bulbs and making weighings. The foregoing procedure was repeated day and night continuously during the entire pupal period of each animal.

The results of the experiments are shown graphically in Figures 6–10, inclusive. The rates of oxygen consumption are indicated by dots, the rates of carbon-dioxide production by circles, and the respiratory quotient (CO_2/O_2) by means of crosses. In Figures 6 and 7 these values are shown for two different males, and in Figures 8

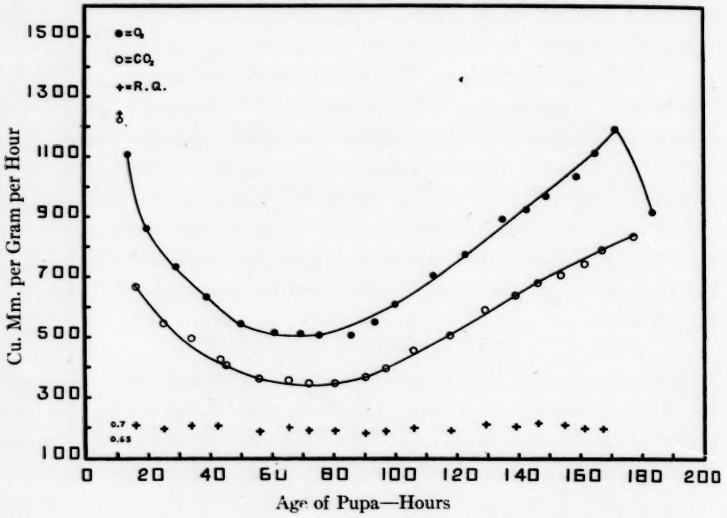

FIG. 6.—Oxygen consumption, carbon-dioxide production, and respiratory quotient of male bee-moth pupa. Temperature, 30° C.

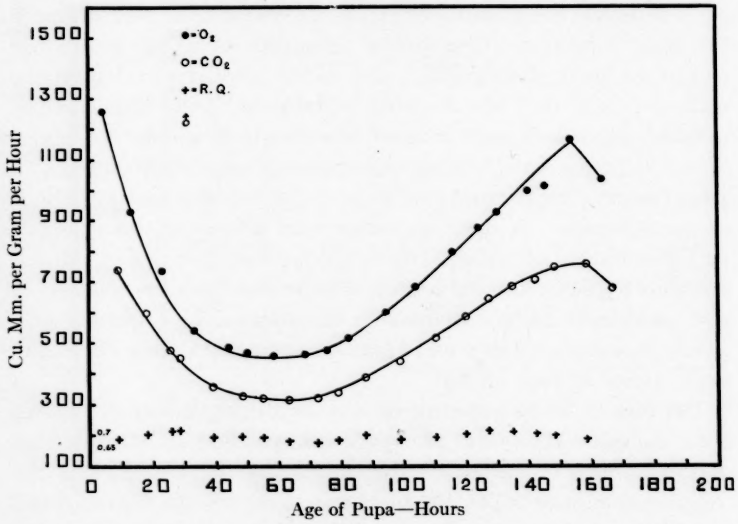

FIG. 7.—Oxygen consumption, carbon-dioxide production, and respiratory quotient of male bee-moth pupa. Temperature, 30° C.

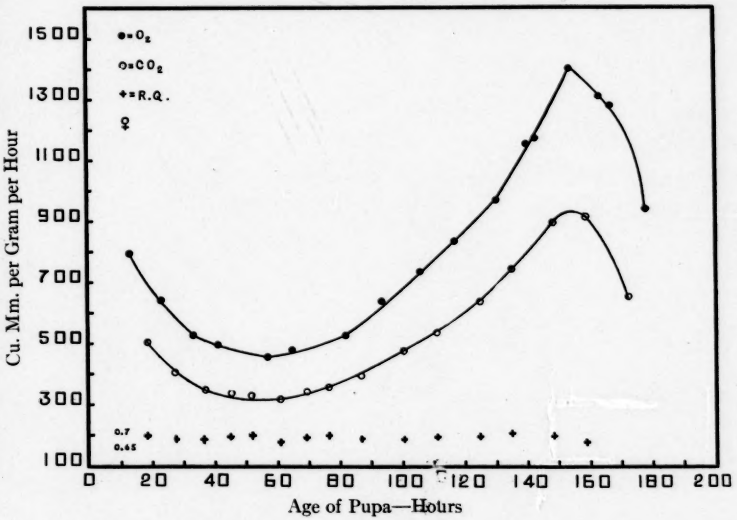

FIG. 8.—Oxygen consumption, carbon-dioxide production, and respiratory quotient of female bee-moth pupa. Temperature, 30° C.

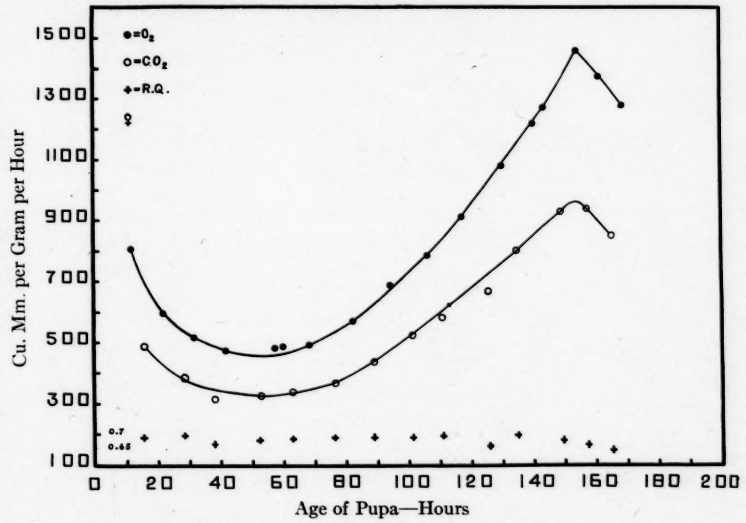

FIG. 9.—Oxygen consumption, carbon-dioxide production, and respiratory quotient of female bee-moth pupa. Temperature, 30° C.

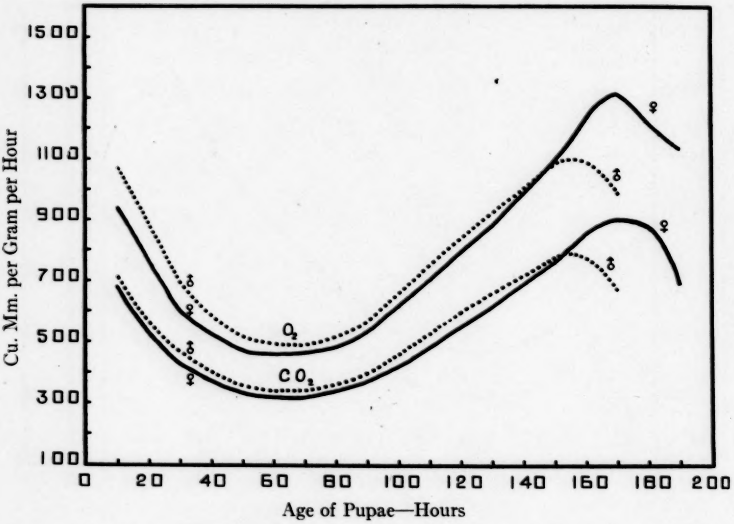

FIG. 10.—The upper dotted curve is the composite oxygen-consumption curve; and the lower dotted curve, the composite carbon-dioxide-output curve for 9 male bee-moth pupae. The upper solid curve is the composite oxygen-consumption curve; and the lower solid curve, the composite carbon-dioxide-output curve for 10 female bee-moth pupae. Temperature, 30° C.

TABLE II

First four columns represent cubic millimeters per gram of pupa per hour.

	Oxygen		Carbon Dioxide		Average Respiratory Quotient throughout Pupal Period
	Minimum	Maximum	Minimum	Maximum	
Male Pupae—Nos. 49–57, Inclusive—9 Individuals					
Average of group	494	1,205	336	851	0.696
Lowest of group	450	1,120	300	770	0.682
Highest of group	530	1,280	365	940	0.705
Female Pupae—Nos. 58–67, Inclusive—10 Individuals					
Average of group	458	1,396	312	969	0.694
Lowest of group	430	1,230	295	860	0.681
Highest of group	500	1,550	335	1,080	0.713

and 9 for two different females. The graphs shown have been selected as typical. The composite oxygen consumption and composite carbon-dioxide output curves are represented in Figure 10 for the 9 male and 10 female pupae used in this part of the work. The points on the curves in Figure 10 represent arithmetical averages.

The carbon-dioxide curves parallel the oxygen-consumption curves rather closely, and in Figure 10 they indicate the same sort of sex difference found for oxygen consumption. The oxygen curves obtained in this part of the work are in agreement with those reported earlier in this paper. Both oxygen and carbon-dioxide curves indicate that the female pupae have the lowest minimum and the highest maximum rates. This is further indicated in Table II, which shows a summary of the minimum rate and the maximum rate of oxygen consumption and carbon-dioxide production of the pupae used in this part of the work. The average respiratory quotient is shown in this table, and also the lowest and highest value obtained for the individuals.

SEX DIFFERENCE

The cause of the sex differences reported above is not evident. Krogh has suggested that in pupae the rate of metabolism is proportional to the amount of organized tissue present and capable of actively respiring. If this assumption is correct, the sex difference may possibly be due to a greater amount of organized tissue being present in the male during most of the pupal period, while toward the end of the pupal stage the females may have a relatively larger amount of active organized tissue than the males. This might account for the higher rates of the males during the major part of pupal development and the higher rates on the part of the females near the end of the pupal stage.

Although the pupae were quiescent and under basal metabolic conditions during the course of these experiments, it is not considered that the rates of oxygen consumption and carbon-dioxide output reported here represent basal metabolic rates. It is impossible at present to calculate the rates of respiratory exchange on the basis of the actual amount of actively respiring tissue present in each pupa, on account of the processes of histolysis and histogenesis which prob-

ably proceed at different and varying rates. Instead, the rates were calculated on the basis of a unit amount (1 gm.) of entire pupa in which the relative amounts of organized and unorganized tissue vary from time to time throughout metamorphosis. Whether or not there exists a true basal metabolic sex difference has not been determined. At the time of emergence the female moths seem to be more mature sexually than the males, and this points to the possibility that the females might actually have relatively more organized tissue than the males near the end of the pupal period.

Evidence of the existence of a sex difference in the rates of respiratory exchange has been reported previously by Fink (1925) for certain pupae, notably *Leptinotarsa decemlineata* and *Popillia japonica*. Other workers, including Clare (1925) and Frew (1929), have found no such difference in the species which they investigated.

THE RESPIRATORY QUOTIENT

In *Galleria mellonella* throughout the pupal period the respiratory quotient remains quite close to 0.69, as in Figures 11–14, inclusive. This is true for each of the 19 pupae upon which the determinations were made. Other workers have usually found the values of the respiratory quotient to show considerable variation from time to time during pupal life. Rather low values have usually been reported for the respiratory quotients of pupae; for example, Weinland (1905) obtained for *Calliphora* pupae an average respiratory quotient of 0.71 and lower. Other references are furnished by Needham (1929) and Sayle (1928), who have reviewed the literature dealing with various phases of insect metamorphosis.

The underlying cause of the fairly constant respiratory quotient with an average value of 0.69 found for *Galleria mellonella* pupae is a matter of uncertainty. In attempting to interpret the meaning of this value, the following quotation from Cathcart and Markowitz (1927) seems to have special significance. "The fact is only too frequently not appreciated or, if appreciated subconsciously, is ignored in practice that the CO_2/O_2 ratio represents not a single, relatively simple, physiological phenomenon but is the sum of an infinitely large, relatively unknown series of phenomena." On first thought a

quotient of 0.69 might appear to be indicative of a practically exclusive oxidation of fat; but probably the explanation is not quite so simple, as the oxidation of fat in animals is usually accompanied by, and dependent upon, the oxidation of carbohydrate. Moreover, the oxidation of fat would not explain the very low quotients reported by some workers for certain pupae. It is recognized that the conversion of carbohydrate into fat, or of fat into carbohydrate, and other chemical transformations in the animal body will modify the value of the respiratory quotient. Richardson (1929) has published an excellent discussion of the respiratory quotient and the factors which modify it. In the case of *Galleria mellonella* pupae the writers are of the opinion that a probable explanation of the respiratory quotient obtained is that the fat stored up in the fat body during the larval stage is converted during the pupal period into glucose and oxidized as carbohydrate. The conversion of fat, relatively poor in oxygen, into carbohydrate, relatively rich in oxygen, would tend to produce a drop in the value of the quotient. That this is true is indicated by the low respiratory quotients obtained for hibernating animals which utilize the fat previously stored up. Bodansky (1930) states that in such animals the glycerol derived from fat metabolism is probably converted at least in part into sugar. It is well known that when sugar chiefly is being oxidized in the animal body, the respiratory quotient tends to be unity. While it is realized that the respiratory quotient may be the resultant of several, perhaps many, different processes taking place in the pupae simultaneously, it is conceivable that the value of the respiratory quotient obtained for *Galleria* pupae may be due mainly to a conversion of fat to glucose and the oxidation of this carbohydrate. Biochemical analyses of *Galleria* pupae in different stages of development are in progress, and it is hoped that these will show the true cause of the value of the respiratory quotient which has been obtained. A fine discussion of the chemical changes during the metamorphosis of insects has been published by Needham (1929). Further careful biochemical analyses will be necessary before we can be sure of the nature of the fundamental chemical transformations accompanying the metamorphosis of insects.

SUMMARY AND CONCLUSIONS

1. Measurements of oxygen consumption and carbon-dioxide production have been made on individual *Galleria mellonella* pupae practically continuously throughout pupal development. The rates are high at first, then decrease steadily to a minimum, rise steadily to a maximum, and finally decrease considerably just before emergence.

2. The male pupae have the higher rates during approximately the first 150 hours (at 30° C.), while during the approximately 40 remaining hours of pupal development the females have rates considerably higher than the males. A significant sex difference exists between the maximum rates of the females and the maximum rates of the males; these rates are higher for the females. This is probably not a sex difference in basal metabolism but may be due to a sex difference in the relative amounts of actively respiring tissue present in the male and female pupae at corresponding periods of pupal development.

3. Throughout pupal development the respiratory quotient remains fairly close to the average value of 0.69. A suggested explanation of this value is that it is due mainly to synthesis of glucose from fat and the simultaneous oxidation of the glucose in the pupae.

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A STUDY OF THE CONDITIONS OF ENCYSTMENT OF COLPODA CUCULLUS

(Four figures)

H. ALBERT BARKER AND C. V. TAYLOR
University of Chicago

THE ciliate *Colpoda cucullus* is conspicuous for the readiness with which it forms cysts. Figure 1 illustrates how the organism multiplies and then encysts when inoculated into $\frac{1}{3}$ cc. of a hay infusion. It will be seen that during the first 3 days after inoculation the total number of organisms increases rapidly while the cysts remain very few. On the fourth day, however, there is no further increase in the total number of organisms; instead of multiplying, as during the previous days, at least 90 per cent of the ciliates encyst.

The object of this investigation has been to determine what environmental factors may induce this striking change. What influences the organisms to encyst at the particular time that they do?

It is unnecessary to discuss the literature of encystment in detail since Kater and Burroughs (1926) and Darby (1929) have reviewed the opinions of the various investigators somewhat fully. Moreover, there would be little profit in so doing because the previous work is confused and unconvincing, owing chiefly to the attempt to compare the encystment of widely different organisms and, from the point of view of the experimentalist, owing to the failure adequately to recognize the great complexity of the protozoan environment.

Opinions have gradually developed that one of several environmental factors is important for encystment: either food supply (Stolte, 1922) or the hydrogen-ion concentration of the medium (Darby, 1929) or the accumulation of excretion products (Mast and Ibara, 1923). Koffmann (1924) suggests that the hydrogen-ion concentration is very important in the encystment behavior of *Colpoda cucullus*, but he offers no reliable evidence. Two other factors, oxygen tension (Rhumbler, 1888) and bacterial flora (Fabre-Domergue,

1888), have been mentioned, but apparently neither has been experimentally investigated.

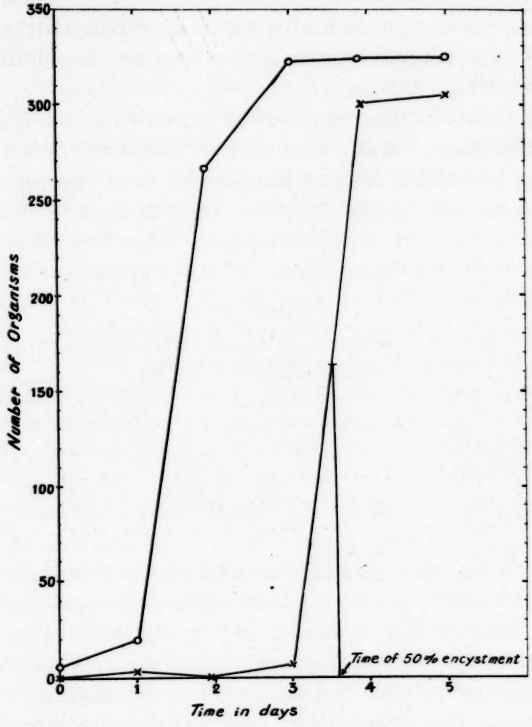

FIG. 1.—Showing the encystment behavior of *Colpoda* in ½ cc. of hay infusion. *o* indicates total number of organisms; *x* indicates number of cysts.

CULTURE OF THE ORGANISMS

Previous investigators studying the encystment behavior of *Colpoda* have used the classical hay infusions as media. But hay infusions are singularly unsuitable as media with which to conduct this sort of experimental investigation. This is true because their composition is unknown and uncontrollable, and doubtless varies greatly as prepared at different times and places. Thus, the buffered infusions of Koffmann (1924) always showed a rapid rise of alkalinity

to a pH of over 9, whereas the hay infusions prepared in this laboratory seldom became more alkaline than a pH of 8. This indicates a large difference either in composition or in bacterial flora, or both. Furthermore, for experimental work it is evident that a medium should be as simple and reproducible as possible. Hay infusions fulfil neither of these demands.

The bacterial situation in infusions is particularly complex. One may readily show that of the numerous distinct types of bacteria which may be isolated from an infusion not more than one or a few may serve as food for the Protozoa. Indeed, most of the bacteria will have a more or less toxic influence upon the Protozoa. Further, the appearance and disappearance of both favorable and unfavorable bacteria will occur at random as a result of chance contaminations and will not be under the control of the investigator. Thus one would be unjustified in assuming that throughout a large series of experiments the conditions in an infusion would remain comparable.

TABLE I

BALANCED PHYSIOLOGICAL MEDIUM (AFTER OSTERHOUT, 1906)

(6 per cent total salt [0.937 M])

0.937 M	Parts
$NaCl$	1,000
$MgCl_2.6H_2O$	78
$MgSO_4.7H_2O$	38
KCl	22
$CaCl_2$	10

For these reasons hay infusions were discarded for these investigations. Instead, the reproducible, non-nutritive medium shown in Table I, supplemented by the addition of a pure strain of bacteria for food, was used.

This solution was diluted five hundred times to the approximate concentration of pond water, or 0.012 per cent total salt (0.0018 M). One cc. of M/20 NaH_2PO_4 was added to each 30 cc. of this diluted solution for buffering, and the pH adjusted with suitable quantities of M/20 NaOH.

Colpoda in this medium were fed upon suspensions of a pure culture of the bacterium *Pseudomonas fluorescens*. This bacterium is eminently suitable as a food for *Colpoda*. When grown in suspen-

sions of it, they appear large and well fed, their division rate is equal to or greater than that in the most favorable hay infusions, they continue normally active and can be kept indefinitely on this single diet without degeneration.

Pseudomonas fluorescens may be isolated from soils by the use of a liquid enrichment culture in which 2 per cent asparagine serves as the only source of carbon and nitrogen. The bacterium may be isolated and grows rapidly on ordinary yeast—or peptone-glucose-agar plates at 37° C. or below. If inoculated heavily on to such plates, the colonies spread out within a few days into a continuous sheet from which masses of bacteria may be removed with a sterile platinum loop in order to be fed to the Protozoa.

The density of the bacterial suspension provided for the Protozoa is difficult to define except as that density in which optimal growth occurs. If too many bacteria surround the Protozoa, the latter are adversely affected as shown by a strongly reduced division rate. Definitely pathologic forms may even appear. In an exceedingly dilute suspension of bacteria the Protozoa show reduction in size and division rate. In short, they behave like any undernourished organisms. Between these two extremes is a relatively wide range of bacterial suspension densities in which the division rate and the size of the *Colpoda* are maximal and approximately constant.

This most favorable density of bacterial suspension will of course depend also in part upon the number of Protozoa inoculated into a given volume of the medium. Whereas 500 *Colpoda* might ingest all of the bacteria in a given suspension, which was originally too dense, thus making the medium quite clear in 24 hours, a single organism could have no appreciable effect upon the density and might be lost.

An approximate idea of a favorable suspension density may be gained from the following illustration: the mass of bacteria which will fill a platinum wire loop 1 mm. in diameter is sufficient to make 6 suspensions of $\frac{1}{2}$ cc. into each of which 20 *Colpoda* are to be inoculated.

No effort was made to avoid air contamination of the *Pseudomonas* suspensions. It was assumed that the suspensions would not be sufficiently nutritive to permit the growth of contaminating

micro-organisms. Justification for this may be seen in the fact that molds were never observed to develop during the experiments.

METHOD

The experiments all consisted in following the reproduction and encystment of *Colpoda* in a given volume of medium by making daily counts of the number of free living and encysted individuals.

Ground-glass protozoan isolation dishes were used as containers. These permit of the organisms being conveniently counted with the aid of a low-power dissecting microscope and a mouth pipette.

In the dishes was placed $\frac{1}{2}$ cc. of the buffered salt medium already described. This same volume was used in all of the experiments. To this was added bacteria, when these were used, by means of a sterilized platinum wire loop. A definite number of free swimming *Colpoda* were then added.

The hydrogen-ion concentration of the original salt medium in any experiment was determined to within ± 0.1 pH by comparing colorimetrically several cubic centimeters of it with commercial standards. The pH at the end of the experiments was measured with approximately the same degree of accuracy by colorimetric comparison of the unknown and commercial standards in vials of somewhat less than $\frac{1}{2}$ cc. capacity.

The *Colpoda* used were all members of a single clone and, at the beginning of each set of experiments, were similar in that they were taken from a culture in which all the organisms had been permanently encysted and had, just previous to the experiment, been stimulated to excyst by the application of hay infusion. Such recently excysted organisms tend to be uniform in size. They were washed at least twice to remove traces of hay infusion before being inoculated into the experimental dishes.

Decrease in the volume of medium by evaporation during the experiment was reduced to a minimum by stacking the culture dishes in a moist chamber.

All experiments were carried out in a constant temperature room at 72° F.

EXPERIMENTAL

a) Normal growth-encystment curve of "Colpoda."—The points in Figure 2 connected by the broken lines were obtained by counting

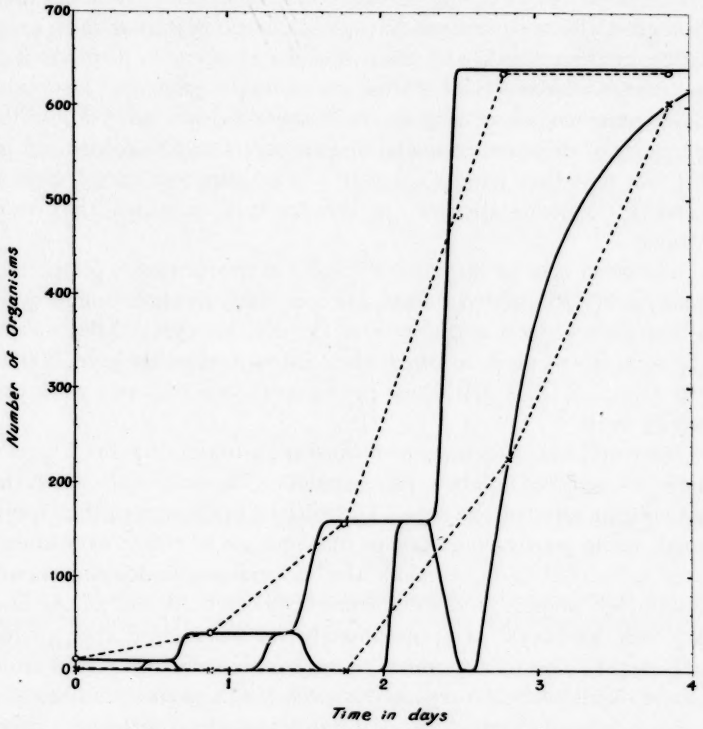

FIG. 2.—The normal time-number relations of *Colpoda* in ½ cc. of a bacterial suspension. *o* indicates observed number of organisms (free and cyst); *x* indicates observed number of cysts; solid line (upper) indicates actual total number of organisms; solid line (lower) indicates actual number of cysts.

each day both free swimming organisms and cysts in a dish containing ½ cc. of a bacterial suspension originally inoculated with 5 *Colpoda*. At the beginning of the experiment the pH of the medium was 6.2. At its conclusion, when almost all of the Colpoda had encysted, the pH had risen to 6.6.

The broken-line curves of Figure 2 give only an inaccurate picture of the time-number relations in such an experiment. This may be made clear by considering the following factors:

Encystment in *Colpoda* is of two distinct types. Preceding division, this ciliate always encysts. Within the cyst it divides once, twice, or three times, and then immediately excysts to produce 2, 4 (most commonly), or 8 free swimming organisms. Rhumbler (1888) applied to such cysts the name "division cysts." For the purposes of these experiments, division cysts will be recognized by the fact that they endure for only a few hours and excyst spontaneously, following division, in the medium in which they were formed.

The other type of encystment results in the formation of "permanent cysts" (Rhumbler). These are commonly single or double cysts of the same general appearance as the division cysts. They do not excyst in the medium in which they are formed unless given a definite stimulus. It is with these permanent cysts that this work primarily deals.

Since division cysts must be formed prior to cell division, Figure 2 may be corrected to show this behavior. The solid lines show the actual time-number relations. The initial 5 organisms in the experiment, being poorly nourished in consequence of recent excystment from permanent cysts, remain in the bacterial suspension some hours before they prepare to divide. Some time before the end of the first day they all encyst at approximately the same time, then divide and excyst. The total number of organisms suddenly jumps from 5 to 20. This process is repeated a second, a third time, until finally increase in total numbers stops. From this time on permanent cysts form until eventually free living forms disappear from the culture. This typical time-number relation is taken as the basic point of departure for the subsequent experiments.

b) The effect of hydrogen-ion concentration on encystment.—One group of experiments was designed to test whether or not *Colpoda* could be kept from permanent encystment indefinitely, within a wide range of hydrogen-ion concentrations, by frequently transferring the organisms.

Bacterial suspensions of 5 different initial pH's were used. In each

dish the initial 10 organisms were allowed to reproduce for 2 days, counts of motile and cyst forms being made daily. At the end of 2 days 10 motile *Colpoda* were transferred to a new dish having the same pH as that of the original. These transfers were repeated at 2-day intervals for 8 days. Figure 3 shows the complete behavior for a single set of transfers made in media of pH range 6.2–6.6. The data of all the experiments are summarized in Table II by averaging for each set the number of motile organisms and cysts present at the end of the first and second days after each transfer.

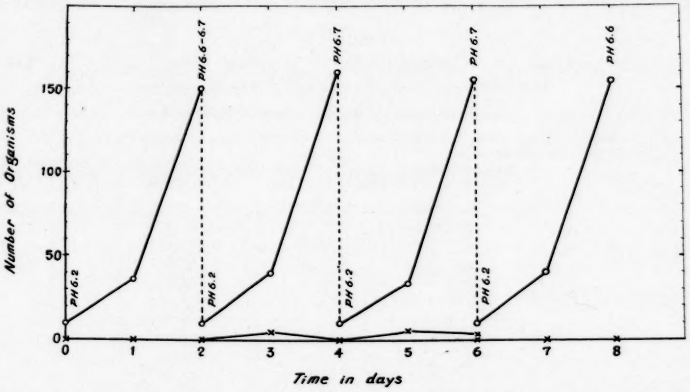

FIG. 3.—Showing the time-number relations during successive transfers into ½ cc. of bacterial suspension in the range of pH 6.2–6.7. *o* indicates total number of organisms; *x* indicates number of cysts.

These data indicate that the encystment behavior of *Colpoda* in media ranging from pH 6.2 to pH 8.2 is essentially the same. Neither the reproduction nor the encystment of the organisms appears to be markedly influenced by a simple change of hydrogen-ion concentration within these limits. It may be noted that the small percentage of cysts which do occur at the end of the second day are mostly, if not all, division cysts.

Further, we may conclude that *Colpoda* may be prevented from permanent encystment for considerable lengths of time, if not indefinitely, by frequent transfers. In order more conclusively to demonstrate this fact, three parallel series of transfer experiments like

those described above were extended for 21 days. Permanent cysts seldom appeared in these experiments and never constituted more than 10 per cent of the total number of organisms present. From these observations it appears that encystment in *Colpoda* is a phenomenon dependent upon some cumulative factor in its environment; in a frequently renewed environment permanent encystment does not occur.

Although encystment is not induced by any hydrogen-ion concentration in the range studied, yet it may occur with equal rapidity in any part of this range under suitable conditions.

TABLE II

SHOWING THAT THE pH OF MEDIUM DOES NOT IN ITSELF APPRECIABLY ACCELERATE THE FORMATION OF PERMANENT CYSTS IN *Colpoda*

(*f* indicates motile forms; *c* indicates cysts)

Series	Range of pH in 2-Day Intervals	Average No. at End of First Day	Average No. at End of Second Day	Average Per Cent of Cysts at End of Second Day
I	6.2–6.7	37*f*;3*c*	156*f*;1*c*	0.32
	6.8–7.3	38*f*;1*c*	155*f*;4*c*	2.3
	7.2–7.6	35*f*;4*c*	152*f*;6*c*	3.8
	7.6–7.8	38*f*;2*c*	152*f*;3*c*	1.9
	7.9–8.2	38*f*;2*c*	158*f*;2*c*	1.2
II	6.2–6.7	38*f*;2*c*	157*f*;3*c*	1.9
	6.8–7.4	39*f*;1*c*	162*f*;6*c*	3.5
	7.2–7.6	38*f*;2*c*	152*f*;8*c*	5.2
	7.6–7.8	38*f*;1*c*	155*f*;2*c*	1.3
	7.8–8.2	37*f*;2*c*	152*f*;4*c*	2.5

This may be observed by placing 10 *Colpoda* in ½ cc. of the bacterial suspension and allowing them to multiply undisturbed for 3 or 4 days. At the end of this time a large percentage of the organisms will have permanently encysted, as the data of Table III indicate. It will be seen that a high percentage of encystment occurred in these experiments in from 3 to 4 days in all pH ranges.

We have seen, then, that the encystment behavior is not strongly influenced either in a positive sense by facilitating encystment or negatively by inhibiting encystment by the hydrogen-ion concentration of the medium. In the experiments to follow, therefore, slight variations of pH may be disregarded as not contributing to the encystment behavior observed.

c) *The effect of food upon encystment.*—Experiments were performed which showed that the rapid permanent encystment in the third day, as seen in the typical time-number curve (Fig. 2), was not due to lack or abundance of food or to a direct effect of the bacteria upon the medium.

These experiments were made by comparing the behavior of a number of *Colpoda* (1) in media plentifully supplied with bacteria and (2) in media to which no bacteria were added. In the media with bacteria, the *Colpoda* always appeared large and well fed, and showed in consequence a maximal reproductive rate. In the unbac-

TABLE III

SHOWING THAT THE pH OF THE MEDIUM DOES NOT IN ITSELF APPRECIABLY RETARD THE FORMATION OF PERMANENT CYSTS

SERIES	NO. OF EXPERIMENTS	pH RANGE	LENGTH OF EXPERIMENT	AVERAGE NO. OF ORGANISMS AT END OF EXPERIMENT		PER CENT CYSTS AT END OF EXPERIMENT
				Free	Encysted	
I.....	2	6.2–6.8	3.5 days	38	625	94
	2	6.8–7.0	3.5	6	465	98
	2	7.2–7.8	3.5	65	405	86
	2	7.6–7.9	3.5	0	630	100
	2	8.0–8.4	3.5	3	570	99
II.....	2	6.2–6.6	3.75	27	655	96
	2	6.8–7.3	3.75	20	670	97
	2	7.2–7.8	3.75	7	485	98
	2	7.6–8.0	3.75	60	480	88
	2	8.2–8.4	3.75	0	400	100

terized media, the organisms were invariably small, transparent, and conspicuously undernourished, and they divided at approximately one-half of the maximal rate, i.e., division was twofold instead of fourfold.

The free forms and cysts were counted each day, and the time which elapsed until 50 per cent of the organisms present at any one time had permanently encysted was determined. In order to keep the number of *Colpoda* approximately constant during the experiments, each day the excess of ciliates over that number originally present was removed with a mouth pipette. It is to be noted that only the motile forms, and never the cysts, were removed.

The time of 50 per cent permanent encystment was taken as a

suitable measure of the time of rapid rise in the number of permanent cysts. The time required for complete encystment is not suitable because there frequently appear to be a small percentage of organisms which encyst much less readily than the average. Likewise there are organisms which encyst more readily than the average. The time of 50 per cent encystment was therefore chosen as the most representative measure of the behavior of the ciliates in any one culture. Although the 50 per cent encystment time is used, it is never to be assumed that no more than 50 per cent of the *Colpoda* encysted. On the contrary, the behavior shown in Figure 2 is typical, where, shortly after half the organisms have encysted, the remaining motile forms also encyst.

TABLE IV

SHOWING THAT THE TIME OF ENCYSTMENT IS LARGELY INDEPENDENT OF THE AVAILABLE FOOD SUPPLY

Food	No. of Experiments	Initial pH	Initial No. of Free *Colpoda*	Average No. of *Colpoda*	Time (in Days) of 50 Per Cent Encystment		
					Minimum	Maximum	Average
Abundant bacteria	6	6.8–7.0	10	30.0	4	9	6.0
No bacteria	8	6.8–7.1	10	17.7	6	10	7.7
Abundant bacteria	8	6.9–7.0	100	260	1.8	3.5	2.8
Abundant bacteria	8	6.9–7.0	200	497	1.8	2.8	2.4
No bacteria	5	7.0–7.1	100	159	1.5	3.0	2.2

Table IV summarizes the data. It will be seen that those organisms which were undernourished required approximately the same time to encyst as did the well-fed organisms. In fact, the undernourished *Colpoda* take slightly longer to encyst than do the better-fed individuals. Evidently diminution of food supply is not what causes the rapid formation of permanent cysts on the third and fourth days in the typical time-number curve experiment (Fig. 2).

Furthermore, the bacteria do not of themselves bring about changes in the media which induce permanent encystment. This may be seen by comparing the number of days required for 50 per cent encystment in those bacterized cultures started with 10 and with 100 and 200 ciliates. To both the cultures having few and those having many initial organisms, approximately the same quantity of

bacteria was added at the beginning of the experiments. But in the initial 10-organism experiments the encystment time averaged 6.8 days, while in the 100- and 200-organism experiments it averaged 2.5 days. The evidence indicates, then, that not only cultures with different quantities of food may encyst in approximately the same time, but cultures with the same amount of food may encyst in markedly different times.

d) *The effect of crowding upon encystment.*—The data of Table III lead directly to a consideration of what appears to be the most important factor for the encystment of *Colpoda*. In the typical time-number curve experiment, permanent encystment did not occur until after a large increase by fission of organisms in the culture. This increase in concentration of organisms is accompanied by a change in the chemical constitution of the medium due to their metabolism, and in a diminution of the food supply. The two incidental changes in the medium which are easiest to investigate, viz., the food supply and hydrogen-ion concentration, have been shown to be insufficient of themselves to account for the encystment behavior of *Colpoda*. We shall now present the evidence that nevertheless crowding does actually induce permanent encystment, and that in the absence of crowding, permanent encystment may be delayed or prevented altogether.

Much of the data already presented is evidence on the effect of crowding. Figure 3 illustrates one of ten experiments, summarized in Table I, in which no permanent encystment occurs for a period of 8 days, when transfers are made every other day such that the organisms are thus prevented from becoming crowded and markedly altering their environment. A similar transfer experiment is described in which permanent encystment was not observed during 3 weeks. Evidently, permanent encystment need never occur when the cumulative changes in the environment brought about by the Protozoa are avoided by the frequent changing or renewing of their media.

If the media are not renewed, then the rapidity of encystment will depend upon the average number of organisms present in a given volume. The greater the average number of organisms in a volume of medium, the less will be the time required for them to alter their

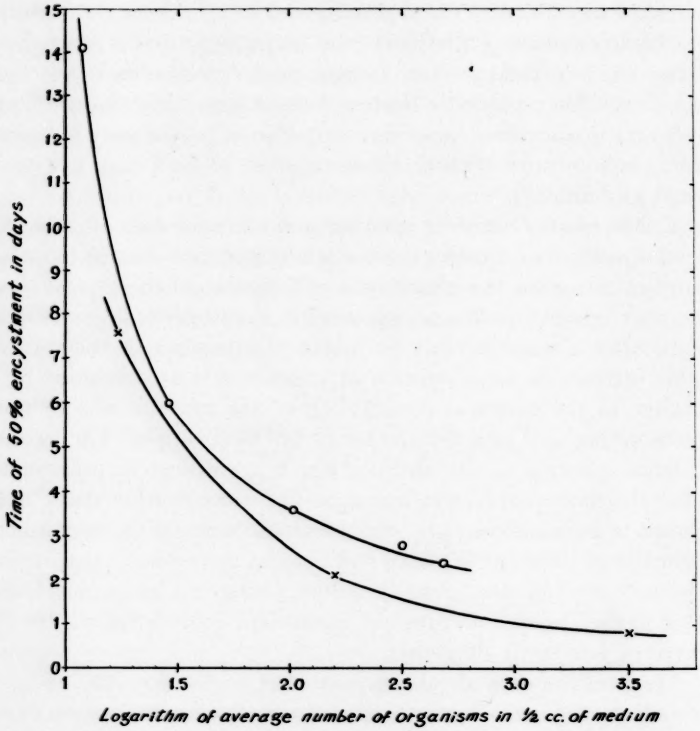

FIG. 4.—Showing the dependence of time of 50 per cent encystment upon crowding of organisms. *o* indicates experiments with bacteria; *x* indicates experiments without bacteria.

TABLE V

SHOWING THE DEPENDENCE OF ENCYSTMENT TIME UPON THE CROWDING OF THE *Colpoda*

FOOD	NO. OF EXPERIMENTS	AVERAGE NO. OF *Colpoda*	TIME (IN DAYS) OF 50 PER CENT ENCYSTMENT		
			Minimum	Maximum	Average
Abundant bacteria.....	6	12.5	9.0	17.0	14.1
Abundant bacteria.....	6	30.0	4.0	9.0	6
Abundant bacteria.....	10	105	2.8	5.0	3.6
Abundant bacteria.....	8	260	1.8	3.5	2.8
Abundant bacteria.....	8	497	1.8	2.8	2.4
No bacteria.....	8	17.7	6.0	10.0	7.7
No bacteria.....	5	159	1.5	3.0	2.2
No bacteria.....	40	1,800+	.....	<1	<1

environment to the extent that they be induced to encyst. Table V summarizes the data on the effect of concentration of organisms on the time of 50 per cent encystment in cultures of the same volume ($\frac{1}{2}$ cc.). Figure 4 illustrates graphically the dependence of encystment time upon concentration of organisms. From these data it may be concluded that crowding is of primary importance for the encystment of *Colpoda*.

DISCUSSION

Although the evidence presented strongly indicates the primary importance of crowding and the alteration of the environment due to the accumulation of metabolic products, it should be emphasized that we need not therefore conclude that these are the only factors operating to induce encystment. Figure 4 suggests indeed that *Colpoda* without food may encyst in a slightly shorter time than do well-fed organisms. But food is evidently a factor of quite secondary importance. Also, it may be true that hydrogen-ion concentrations on the borders of the physiological range influence encystment, as has been suggested by previous workers. Further, in the course of this investigation it has been observed that in a bacterial suspension so dense and unfavorable that pathological forms appear, those *Colpoda* which do survive the extreme condition encyst. Yet even while recognizing these other possible factors of encystment, we must repeat that under otherwise favorable conditions encystment is a phenomenon dependent upon a cumulative alteration of the environment by the *Colpoda* themselves.

As to the physical identity of the environmental change which induces encystment we have no evidence. It seems improbable that it is a change in either O_2 or CO_2 tension, for the culture dishes used were such as to allow rapid diffusion and near-equilibrium of the media with the air at all times. More probably some non-volatile product of metabolism is concerned in the effect.

SUMMARY

1. A method of culturing *Colpoda cucullus* on a suspension of a pure strain of bacteria in a non-nutritive medium is described.

2. Permanent encystment may be prevented for a long time, and presumably indefinitely, by frequently renewing the culture medium and not allowing the organisms to become crowded.

3. The formation of permanent cysts is normally dependent upon a crowding of the *Colpoda* or, where they are not numerous at any one time, upon a cumulative alteration of the medium brought about by the ciliates themselves. The more abundant the *Colpoda* in a certain volume of a given medium, the sooner will permanent encystment occur.

4. Permanent encystment is not markedly accelerated or retarded by the absence or abundance of food.

5. Permanent encystment is not appreciably influenced by the hydrogen-ion concentration of the medium within the limits of pH 6.2–8.2.

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THE ACTION OF THE CHLORIDES OF SODIUM, POTASSIUM, CALCIUM, AND MAGNESIUM ON THE PROTOPLASM OF AMOEBA DUBIA

L. V. HEILBRUNN AND KATHRYN DAUGHERTY
University of Pennsylvania

IT IS scarcely necessary to discuss the importance of salts in protoplasm. One of the essential characteristics of the living substance is the fact that it contains salts of the alkaline and alkaline earth metals. Maintenance of life seems to depend on the proper balance between the sodium, potassium, calcium, and magnesium ions of the cell.

Many workers have studied the antagonistic action of these ions. Perhaps the most work has been done by the muscle physiologists who have been interested in the behavior of heart or skeletal muscle when one ion or another is added or removed. Such study is doubtless important, but it has yielded little or no information concerning the fundamental nature of the antagonism. With the development of the science of colloid chemistry, it has been commonly held that the salts of protoplasm exert effects on the living colloid similar to those which they produce on inanimate colloids. Some have suggested that calcium exerts a precipitating action on the colloids of the cell, and that this effect is antagonized by sodium and potassium ions. This is, indeed, what one might be led to expect if the micellae of the protoplasmic colloid were negatively charged.

Direct studies of the effect of various cations on the colloidal properties of protoplasm have been few. Heilbrunn (1923) showed that calcium and magnesium ions decreased the protoplasmic viscosity of the sea-urchin egg *Arbacia*, and of the protozoan *Stentor*, and that sodium and potassium ions had the opposite effect. Heilbrunn interpreted his results as indicating that the micellae of the protoplasmic colloid bear a positive charge; and there is, indeed, excellent evidence in favor of this view (see Heilbrunn, 1928, chap. x). Cholodnyj (1923) showed independently that calcium ions decrease the protoplasmic viscosity of *Trianea* root hairs and that sodium and potassium ions have the opposite effect. Cholodnyj's

evidence is very clear cut and convincing. Similar results were obtained by Weber (1924) for *Spirogyra* protoplasm. There have also been other papers on the same general subject, and these are reviewed in Heilbrunn (1928). The most widely quoted paper is that of Chambers and Reznikoff (1926). These authors concluded that calcium and magnesium salts increased the viscosity of the protoplasm of *Amoeba dubia*[1] and that sodium and potassium ions had the opposite effect. Their results are therefore in direct contradiction to those of Heilbrunn, Cholodnyj, and Weber.

Now it is, of course, possible that the protoplasm of sea-urchin eggs, *Stentor*, *Trianea*, and *Spirogyra* behaves in one way toward the ions of the alkaline and alkaline earth metals and that the protoplasm of *Amoeba* behaves in just the opposite fashion. The results might even suggest that the micellae of *Amoeba* protoplasm bear a negative charge, whereas those of other types of cells are positively charged. However, as will be shown more fully later, the methods of Chambers and Reznikoff are not very trustworthy, and it was therefore thought advisable to repeat their experiments on *Amoeba*. It has been shown recently (Heilbrunn, 1929*a*, 1929*b*) that the viscosity of the protoplasm of *Amoeba dubia* can be readily determined by the centrifuge method, and for relative viscosity measurements the method is apparently accurate to within 10 or 15 per cent. It is interesting to note that Pekarek's measurement of the absolute viscosity of *Amoeba* protoplasm with a new Brownian movement method shows a very close agreement with Heilbrunn's results. It seems certain that the centrifuge method gives reliable information regarding the viscosity of *Amoeba* protoplasm, and it therefore gives a means of determining the real effect of sodium, calcium, and other ions on this important property of the protoplasm.

With the exception of a few preliminary tests, all the measurements were made by the junior author.

METHODS

The original cultures of *Amoeba dubia* were obtained from Dr. J. A. Dawson, and subcultures were made in accordance with the

[1] In their early work, Chambers and Reznikoff state that their experiments were performed on *Amoeba proteus*, but in a later paper (Reznikoff and Chambers, 1927) they state that all of the work was done on *Amoeba dubia*.

procedure recommended by Dawson (1928). After sufficient practice, it is not very difficult to determine the viscosity of the protoplasm of *Amoeba dubia* by the centrifuge method (see Heilbrunn, 1929*a*, 1929*b*). Amoebae were immersed for varying times in the solutions of the salts to be tested. Following such immersion, tests were made of the protoplasmic viscosity. In each case, the viscosity of the protoplasm of the exposed amoeba was compared with the viscosity of the protoplasm of control animals from the identical culture. In any given culture, the amoebae usually behave in a rather constant fashion. Occasional cultures are too variable for experimental purposes. It is not wise to compare animals from different cultures. A slight difference in the size of the crystals in the protoplasm of the amoeba has a marked influence on the speed of movement of the larger granules or crystals under the influence of centrifugal force. In earlier work on the effect of temperature on the protoplasmic viscosity of *Amoeba dubia*, only those cultures were chosen which showed a particular speed of movement of the crystals for a given centrifugal force at a definite temperature. In this way, different cultures could be compared, or the same culture at different periods of time. In the experiments recorded in this paper, no effort was made to secure uniformity between tests on different days for different cultures. In the data presented, therefore, comparison should only be made between the amoebae of a given experiment.

In solutions of potassium chloride, the crystals have a tendency to fall through the body of the amoeba. The settling of the crystals prevents centrifuge tests, for, if the crystals have already moved through the cell, they present the same appearance as after centrifugal treatment. In order to make viscosity tests of the protoplasm of amoebae immersed in potassium chloride solutions, the amoebae must be rotated in such a way as to prevent the settling of crystals. This can be done by exposing the amoebae to the potassium chloride solutions in centrifuge tubes which are continuously rolled or rotated, so that the position of the amoebae with respect to gravity is constantly being changed. Or the exposed amoebae may be agitated in some form of shaking device. The latter method was the one generally adopted, and the agitated amoebae showed no settling of crystals, presumably because they were constantly being turned about.

In the viscosity determinations, an old model Bausch and Lomb centrifuge was used. This was of the same type as that employed in earlier experiments (Heilbrunn, 1929). With an instrument of this type, at 18°, a force of about 128 times gravity typically causes the crystals to move through half the diameter of the cell in 3 seconds. The number of seconds required for such movement of crystals or granules is a relative measure of the viscosity. Thus, if it requires 3 seconds, the viscosity is taken as 3. The absolute viscosity in such a case is really about 2 poises, or 2 times the viscosity of water (Heilbrunn, 1929*b*). In order to determine the viscosity in any given experiment, it is necessary to make a series of determinations in order to discover exactly how long a centrifugal treatment is necessary to produce the specified degree of movement.

The viscosity values recorded in the body of the paper represent the time, in seconds, required for the crystals to move through one-half of the diameter of the cell when the amoebae are subjected to a centrifugal force 128 times gravity. Roughly, these values are $1\frac{1}{2}$ times as great as the absolute viscosity in terms of the viscosity of water.

In order to avoid osmotic effects, only dilute solutions of the salts were used. The amoebae were immersed in m/40 solutions of sodium or potassium chloride, and in m/60 solutions of calcium or magnesium chloride. A few experiments were also tried with m/50 magnesium chloride. This behaved in the same manner as m/60 magnesium chloride. The solutions were made up in water redistilled from a fused silica still. This water, when freshly prepared, has a pH of 7.0, and the pH of the various solutions was never more than a few tenths of a pH unit removed from neutrality. The slight variations of pH seemed to have no effect on the results.

SODIUM CHLORIDE

Chambers and Reznikoff (1926) state that in solutions of sodium chloride the amoebae become quiescent and the larger granules (or crystals) sink to the bottom. With the solutions used, no such sinking of granules or crystals was ever observed at room temperature. Hundreds of tests were made at temperatures up to 24°, and in no case were the crystals observed to sink as Chambers describes.

However, if the amoebae were heated to 30°, the crystals sink very rapidly. Moreover, if the amoebae are immersed in m/5 sodium chloride, there may be a slight sinking of crystals. Such a solution is strongly hypertonic. At normal temperatures, and with dilute solutions, the crystals do not sink.

Table I shows the results of viscosity tests on amoebae immersed in m/40 sodium chloride solutions for varying lengths of time. The

TABLE I

RELATIVE VISCOSITY OF *Amoeba* PROTOPLASM IN CULTURE SOLUTION AND IN m/40 SODIUM-CHLORIDE SOLUTION

Test No.	Temperature	Normal Viscosity	Time of Exposure (Minutes)	Viscosity in m/40 NaCl
1	18°	3	80	5
2	22°	7	130	5
3	22.5°	5	280	7
4	20°	3	116	7
5	18.5°	3	54	5
6	22.5°	6	262	9
7	22.5°	4	40	6
8	22.5°	5	14	7
9	21.5°	3	27	4
10	22°	7	290	9
11	22°	7	185	9
12	21°	3	112	5
13	22.5°	4	27	7
14	24°	3	20	5
15	21°	6	30	8
16	23°	8	40	10
17	24°	3	51	5

Average increase of viscosity equals 41 per cent.

data consistently show a sharp increase in protoplasmic viscosity. Of seventeen tests, or series of tests, only one fails to show such an increase. This is the second test of the series, and was one of the earliest measurements made. For this reason it is probably not as reliable as the later tests. With increasing use of the centrifuge method on *Amoeba dubia*, one attains a greater degree of proficiency.

Each line of Table I is the summarized result of a series of tests on the effect of sodium chloride on a given culture of amoeba. The exposure time, in the fourth column, is the time at which the definitive test was made. It will be noted that the normal viscosity varies

from culture to culture. This is in part due to differences in temperature. It is also due to differences in the amoebae. Size of crystals varies from one culture to another. The larger the crystals, the more readily they move. The relative viscosity values given in the third column of the table are only to be compared with the corresponding values in the fifth column, and not with each other.

The table shows clearly that dilute sodium chloride solutions caused a decided increase in the viscosity of the protoplasm of *Amoeba dubia*. This increased viscosity is no doubt partly responsible for the sluggish behavior of the amoebae in sodium chloride solutions. The movement of such amoebae is very slow. But the increased viscosity is probably not the only factor involved in producing this retardation in speed of amoeboid movement, for it is very likely that in sodium chloride the forces responsible for the movement are diminished.

POTASSIUM CHLORIDE

When amoebae are immersed in an m/40 potassium chloride solution, movement becomes slow and eventually almost ceases. Presumably as a result of this cessation of movement, the crystals of the protoplasm sink to the bottom of the cell. The protoplasmic viscosity of *Amoeba dubia* is so low, and the crystals of the protoplasm are so large, that they would, of necessity, fall if they were not continually stirred up by the movements of the protoplasm. In amoebae exposed for some time to potassium chloride solutions, ordinary centrifuge tests are not possible, for the crystals or granules are already massed at one end of the cell. It is therefore necessary to prevent the fall of the crystals during exposure to potassium chloride solutions. This can easily be done by keeping the amoebae agitated in the solution so that no one side remains down for any length of time. The amoebae were placed in centrifuge tubes containing potassium chloride solution, and these were then rotated or agitated until it was thought advisable to make a centrifuge test. Usually the agitation was accomplished with the aid of a simple shaking-machine.

Table II shows the results of viscosity determinations of amoebae immersed in potassium chloride solutions and agitated during the

whole period of their exposure. In all, twelve series of tests were performed; and in every instance it was found that the potassium chloride produced an increase in viscosity which was easily measured. This viscosity increase was not as great as in the case of sodium chloride. As before, the exposure time is the time which had elapsed when the definitive test was made.

TABLE II

RELATIVE VISCOSITY OF *Amoeba* PROTOPLASM IN CULTURE SOLUTION AND IN m/40 POTASSIUM CHLORIDE SOLUTION

Test No.	Temperature	Normal Viscosity	Time of Exposure (Minutes)	Viscosity in m/40 KCl
1	22.5°	6	28	8
2	22.5°	4	17	5
3	22.5°	5	96	6
4	21.5°	3	23	4
5	22°	7	28	8
6	22°	7	42	8
7	21°	3	52	4
8	22.5°	4	22	5
9	20.75°	4	54	5
10	23°	8	17	9
11	23°	2	19	3
12	24°	3	30	4

Average increase of viscosity equals 21 per cent.

MAGNESIUM CHLORIDE

The results so far indicate that the monovalent ions, sodium and potassium, cause an increase in the protoplasmic viscosity of the *Amoeba*. It is easy to prove that the bivalent ions, magnesium and calcium, have the opposite effect. Table III shows the results of experiments with magnesium chloride solutions. The amoebae were immersed in an m/60 solution. Owing to the fact that magnesium chloride dissociates into three ions, an m/60 solution of magnesium chloride is approximately equal in osmotic strength to an m/40 sodium chloride solution. Actually, it is slightly weaker, as freezing-point determinations show; but with all these solutions the dilution is so great that there are probably no osmotic effects at all. A few experiments were tried with m/50 magnesium chloride, which is slightly stronger osmotically than an m/40 sodium chloride solution. The m/50 magnesium chloride solution gave exactly the same re-

sults as the weaker solution. The results of immersion in magnesium solutions are clear cut. In every case, there is a very appreciable decrease in protoplasmic viscosity.

TABLE III

RELATIVE VISCOSITY OF *Amoeba* PROTOPLASM IN CULTURE SOLUTION AND IN MAGNESIUM CHLORIDE SOLUTION

Test No.	Temperature	Normal Viscosity	Time of Exposure	Viscosity in m/60 $MgCl_2$	Time of Exposure (Minutes)	Viscosity in m/50 $MgCl_2$
1	24°	3	27	2		
2	21.5°	5	36	4		
3	21°	4	76	3		
4	22°	5	28	4		
5	21°	5	148	3	135	3
6	20°	6	250	4	257	4
7	20.5°	5	184	3	194	3
8	21°	6	197	3	202	3
9	20°	4	132	3	140	3

Average decrease of viscosity equals 33 per cent.

CALCIUM CHLORIDE

Calcium chloride solutions also produce a viscosity decrease. This is shown in Table IV. In every one of seventeen series of

TABLE IV

RELATIVE VISCOSITY OF *Amoeba* PROTOPLASM IN CULTURE SOLUTION AND IN m/60 CALCIUM CHLORIDE SOLUTION

Test No.	Temperature	Normal Viscosity	Time of Exposure (Minutes)	Viscosity in m/60 $CaCl_2$
1	18°	3	195	2
2	22°	7	210	3
3	23°	6	125	2
4	22.5°	5	198	3
5	20°	3	15	2
6	18.5°	3	10	2
7	22.5°	6	337	5
8	22.5°	4	10	3
9	21.5°	3	12	2
10	22°	7	263	5
11	21°	3	60	2
12	22.5°	4	18	3
13	24°	3	34	2
14	21°	6	12	5
15	23°	8	209	4
16	24°	3	37	2
17	22°	5	40	4

Average decrease in viscosity equals 35 per cent.

experiments, exposure to calcium chloride produced an easily measurable decrease in viscosity. The average decrease was 35 per cent. This compares with a 33 per cent decrease observed in the same concentration of magnesium chloride. The difference is hardly significant. But it should be noted that, on the average, the exposures to magnesium ion were longer and that these longer exposures produced a greater effect. In the eight experiments with calcium chloride in which amoebae were exposed for an hour or longer, the average viscosity decrease was 42 per cent, as compared with a viscosity decrease of 26 per cent for exposures of less than an hour. In the experiments with m/60 magnesium chloride, the amoebae exposed for an hour or longer showed an average viscosity decrease of 37 per cent. It is not improbable that calcium chloride has a more pronounced liquefying action than magnesium chloride, but this can hardly be regarded as proved.

It may be regarded as certain, however, that both magnesium and calcium chloride solutions have a marked effect in lowering the viscosity of the protoplasm of *Amoeba dubia*.

AMMONIUM CHLORIDE

It might reasonably be expected that the ammonium ion would act like the sodium and the potassium ion in causing an increase in protoplasmic viscosity. It does produce this effect on the protoplasm of *Arbacia* eggs and *Stentor*. But experiments with *Amoeba* soon showed that after exposure to an m/40 ammonium chloride solution, the protoplasm became markedly more fluid. Ten series of tests on amoebae immersed in ammonium chloride solution indicated a viscosity decrease of 68 per cent after immersion. It was then noted, however, that the crystals had a tendency to sink in the amoebae exposed to ammonium chloride solution, so that they had already begun to move before the centrifuge tests were begun. It was necessary, therefore, to rotate or agitate the amoebae as in the previous experiments with solutions of potassium chloride. Table V shows the results of tests with amoebae which were kept in motion during the time of exposure to the ammonium chloride solution. These tests showed an average decrease in viscosity of 34 per cent.

The unexpected behavior of ammonium chloride solutions is prob-

ably due to a secondary effect. Ammonium chloride in solution tends to hydrolyze into ammonium hydroxide and hydrochloric acid. The ammonium hydroxide penetrates into living cells much more readily than the acid, and it is for this reason, according to Jacobs (1922), that plant and animal cells immersed in solutions of ammonium chloride become alkaline. Jacobs experimented with starfish eggs. Amoebae also tend to become alkaline in ammonium chloride solutions. Thus some amoebae were stained in a dilute neutral red solution (1 part in 1,250,000), and were then immersed in m/40 ammonium chloride solution. Other amoebae stained in the same

TABLE V

RELATIVE VISCOSITY OF *Amoeba dubia* IN CULTURE SOLUTION AND IN AMMONIUM CHLORIDE SOLUTION

Test No.	Temperature	Normal Viscosity	Time of Exposure (Minutes)	Viscosity in m/40 NH_4Cl
1	21.5°	5	22	3
2	21°	3	60	2
3	21°	7	31	5
4	22°	4	82	3
5	22.5°	4	45	3
6	21°	6	100	5
7	21.5°	4	125	2
8	21°	6	155	3
9	21°	3	140	2
10	21°	5	95	3

Average decrease in viscosity equals 34 per cent.

fashion were immersed in m/40 sodium chloride solution as a control. After 2 hours, the amoebae in the ammonium chloride solution had lost their red color, whereas the amoebae in sodium chloride solution were still red after 5 hours. In the amoebae exposed to ammonium chloride solution, the indicator had apparently changed to yellow. The yellow color was faint and hard to recognize. One could be sure of it in the nucleus but not in the cytoplasm. It seems certain, however, that ammonia or ammonium hydroxide had actually penetrated into the cell. The presence of this ammonia or ammonium hydroxide might in itself cause a drop in protoplasmic viscosity. At any rate, in the egg of the sea urchin *Arbacia*, Barth (1929) showed that immersion in dilute solutions of ammonium hydroxide caused a marked decrease in protoplasmic viscosity.

DISCUSSION

The experiments show clearly that sodium and potassium cause an increase in the viscosity of the protoplasm of *Amoeba dubia* and that calcium and magnesium cause a decrease in viscosity. Thus the protoplasm of this *Amoeba* behaves toward these cations in exactly the same fashion as does the protoplasm of *Stentor*, *Arbacia*, *Trianea*, and *Spirogyra*.

How, then, can we interpret the divergent results of Chambers and Reznikoff (1926), which were obtained on the identical form of *Amoeba?* Most of the experiments of these authors were made by injecting solutions of salts into the cells. When a salt solution is injected into a cell, this does not result in the same set of conditions as those which arise when a cell is immersed in a solution of a salt. Suppose, for example, that sodium chloride is injected into an amoeba, and even let us suppose for the sake of argument that the injected solution stays within the cell. The amoeba still remains bathed in a solution which may be rich in calcium and probably is. A cell immersed in a sodium chloride solution is in a medium free of calcium; so that the two sets of conditions are really totally different. There is, thus, no reason to suppose that an amoeba injected with a sodium chloride solution would react in the same way as an amoeba bathed in sodium chloride solution. A priori, one might suppose that if a relatively viscous colloidal solution were mixed with a solution of sodium chloride the viscosity of the colloidal solution would diminish. Thus, if a sodium chloride solution with a viscosity approximately equal to that of water were injected into the protoplasm of *Amoeba*, the viscosity of which is approximately 3–9 times that of water, it is only logical to suppose that the viscosity of the *Amoeba* protoplasm would be lowered by the sodium chloride solution, especially near the point of injection. It might readily be true, therefore, that the injection of a sodium chloride solution would cause some liquefaction of the *Amoeba* protoplasm. Moreover, it is easy to understand why free calcium injected into a cell might produce coagulation. Recent experiments of Heilbrunn have shown clearly that whenever naked protoplasm forms a film about itself the reaction involved is dependent on the presence of calcium ion (see Heilbrunn, 1928, chaps. xiii and xiv). And thus, whereas an injected

sodium chloride solution might be expected to mix freely with the protoplasm of a cell, a calcium chloride solution would produce a film at the point of injection and doubtless other films through the protoplasm. Indeed, the occurrence of films and vacuoles in protoplasm, and the consequent solidifications of the protoplasm, are thought to be largely due to the release of free calcium within the cell. When a cell is immersed in a solution of a calcium salt, the calcium ions enter slowly and are probably, for the most part, bound chemically or physically as soon as they enter.

It is not difficult to understand, therefore, why the results of Chambers and Reznikoff on injected cells differ from those of other authors who studied cells immersed in salt solutions. Experiments in which salt solutions are injected into cells are in a different category from those in which the cells are bathed in the solutions. In our work, no attempt was made to inject the cells.

But it must be remembered that Chambers and Reznikoff also made studies of amoebae immersed in solutions of the chlorides of sodium, potassium, magnesium, and calcium. In these studies, they found that sodium and potassium produced liquefaction, and that calcium and magnesium had a solidifying or coagulative action, just as in the injected amoebae.

Unfortunately, Chambers and Reznikoff's description of their experiments with immersed amoebae is very brief. In their main paper, the effects of immersion in solutions of single salts is described in just one paragraph. It is stated that in solutions of sodium chloride or potassium chloride the amoebae stop moving and their granules sink. They form a clump at the bottom of the cell, and when an amoeba is turned over, the clump remains in its original position. The sinking of the granules is taken as evidence of fluidity. Doubtless, Chambers and Reznikoff made many tests of viscosity with the microdissection needle; but in their description of their experiments, they confine their remarks to a single sentence. In referring to the effect of sodium and of potassium chloride, they say that "upon manipulation with the needle the interior of the quiescent, rounded amoeba is found to be liquid." There is no statement that it is more liquid than in the normal amoeba, but probably this is assumed. Also, one may assume that the sentence quoted

above is true for a large range of concentrations and exposure times, but concentrations and exposure times are not explicitly stated. Concerning the effect of calcium and magnesium salts, we find only the statement that in solutions of these salts the amoebae are active and appear normal as long as they live. There are no observations recorded on the effect of calcium or magnesium salts on the protoplasmic viscosity of amoebae immersed in solutions of these salts.

In attempting to interpret Chambers and Reznikoff's experiments, Heilbrunn (1928) pointed out that the crystals or granules of the *Amoeba* immersed in sodium or potassium chloride solution could not possibly fall in a relatively few minutes, if they fell singly—not even if they were falling through a fluid with no greater viscosity than that of distilled water. Heilbrunn therefore insisted that the essential fact was that sodium and potassium ions caused a clumping of granules. This is indicated also by Chambers and Reznikoff's description of clumping. Now, it is very true that the granules of *Amoeba proteus*, if they fell singly, could not possibly fall as rapidly as Chambers and Reznikoff describe. But Heilbrunn overlooked the fact that in a later paper (Reznikoff and Chambers, 1927) it was pointed out in a footnote that the experiments were performed not on *Amoeba proteus*, as originally stated, but on *Amoeba dubia*. This alters the situation. The crystals or granules of *Amoeba dubia* are much larger, and they might be expected to fall about 10 times as rapidly. Indeed, from the data collected by Heilbrunn (1929*a*, 1929*b*) it can be calculated that the crystals of *Amoeba dubia* would fall through half the diameter of the cell in from 6 to 18 minutes, depending on the temperature. (This is on the assumption that the viscosity of the *Amoeba* protoplasm does not vary with the shearing force, i.e., that the protoplasm behaves as a perfect fluid. If it is not a perfect fluid, then the time of fall would be longer.) One may ask why the granules do not fall in the normal *Amoeba*. The reason is not far to seek. The motion of the *Amoeba* keeps the granules suspended. Presumably, any condition which renders the *Amoeba* quiescent results in a settling of crystals, provided that it does not also produce a rapid coagulation. Thus, the settling of the crystals, as described by Chambers and Reznikoff for amoebae immersed in

solutions of potassium chloride, is essentially due, not to any change in viscosity, but to a stoppage of amoeboid movement.

It should be noted that sodium chloride solutions do not cause any such settling of crystals as Chambers and Reznikoff describe. In solutions of m/10 to m/40 sodium chloride, the *Amoeba* continues to move slowly. This slow movement is probably not enough in itself to prevent a settling of crystals, and it is only the increased viscosity of the protoplasm that prevents it. If, however, the amoebae in the sodium chloride solution are heated to 30°, they stop moving and the crystals settle rapidly. Using Stokes' law, one can calculate the viscosity of the protoplasm of the amoebae in sodium chloride solutions at 30°. Apparently, the viscosity is somewhat greater than the viscosity in culture fluid at 30°, as determined by Heilbrunn (1929*b*). This is again on the assumption that the protoplasm of the *Amoeba* behaves as a perfect fluid.

It seems clear that the conclusions of Chambers and Reznikoff are scarcely justified, and there is every reason to believe that sodium and potassium ions cause an increase in the protoplasmic viscosity of *Amoeba dubia* and that calcium and magnesium ions have the opposite effect. This type of protoplasm, therefore, behaves in the same fashion as sea-urchin eggs, *Stentor*, *Trianea*, and *Spirogyra*.

In all these types of protoplasm we are apparently dealing with a fluid type of material. Now it may be remembered that Heilbrunn (1923) showed that the surface membrane of the cell, which is doubtless a gel, behaved in quite the opposite way from the interior. Calcium and magnesium tend to make the membrane stiffer, and sodium and potassium tend to liquefy it. The protoplasm of *Amoeba dubia* is practically all fluid, and the crystals all move readily when the cell is centrifuged at slow speeds. On the other hand, as was pointed out previously (see Heilbrunn, 1929*a*), there is a stiff cortex in *Amoeba proteus*, and the crystals or granules of this region do not move at all when the cell is centrifuged at slow speeds (this is the plasmagel of Mast, 1926). Now it occurred to us that the cortex or plasmagel of *Amoeba proteus* might have the properties of a cell membrane and that it might behave as does the cell membrane of the sea-urchin egg toward calcium, magnesium, sodium, and potassium ions. Preliminary experiments indicate that this view may be cor-

rect. At any rate, potassium ions cause a very pronounced decrease in the viscosity or plasticity of this cortical ring or material in the *Amoeba proteus*. This is doubtless the explanation of why amoebae round up and become motionless in potassium solutions. Further experiments in this direction are planned. The two types of amoebae, *Amoeba dubia* and *Amoeba proteus*, are both very useful for viscosity studies. With *Amoeba dubia*, it is a simple matter to obtain information regarding the main mass of the protoplasm. With *Amoeba proteus*, one can study the outer gelatinized region which forms the cortex.

Our experiments on *Amoeba dubia* are in perfect agreement with studies on other types of protoplasm, and they indicate that the action of sodium and potassium on the interior of the cell is to cause a viscosity increase, whereas calcium and magnesium have the opposite effect. These facts are of importance for the theory of the antagonistic action of ions. From a physico-chemical standpoint, they are readily understandable if we assume that the micellae of the protoplasmic colloid bear a positive charge; and, indeed, Heilbrunn (1923, 1928) argued from results such as these that the micellae of the protoplasmic colloid actually did bear a positive charge.

We should be inclined to believe, therefore, that the crystals or granules of *Amoeba* protoplasm are charged positively, and that, when this charge is diminished by the replacement of the more potent bivalent cations by the less powerful monovalent cations such as sodium or potassium, there is a consequent tendency for granules to clump, with a resulting rise in viscosity. Actually, there is some evidence from cataphoresis experiments that the micellae of the *Amoeba* protoplasm are charged positively. Such evidence is now being gathered in this laboratory by Mr. Dan, and it is hoped that he will publish shortly.

SUMMARY

1. Specimens of *Amoeba dubia* were immersed in dilute solutions of the chlorides of sodium, potassium, calcium, magnesium, and ammonium, and the protoplasmic viscosity was then determined by the centrifuge method.

2. In sodium chloride the viscosity is increased about 41 per cent.

3. Amoebae immersed in pure solutions of sodium chloride at room temperature continue to move slowly. At 30°, however, they round up and become quiescent. Stoppage of movement at 30° is followed by a fall of the crystals through the protoplasm of the amoeba.

4. In potassium chloride solutions, the viscosity is increased approximately 21 per cent. Potassium causes a stoppage of movement of the amoeba with a consequent fall of crystals through the cell.

5. Magnesium chloride solutions decrease the protoplasmic viscosity by approximately 32 per cent.

6. Calcium chloride causes a decrease in protoplasmic viscosity of about 35 per cent.

7. In ammonium chloride solutions there is an entrance of ammonium hydroxide into the cell. The viscosity is decreased approximately 34 per cent.

8. The results indicate that the protoplasm of *Amoeba dubia* resembles other types of protoplasm in its behavior toward the common cations of living material. The results also support the view that the protoplasmic micellae are positively charged.

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